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THE DAVID GLENDENNING COGAN

Proctor Medal Award

PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

Twenty-third Meeting

San Francisco, California

June 23, 24, and 25, 1954

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David Glendenning Cogan, M.D.

REMARKS MADE ON ACCEPTANCE OF THE PROCTOR MEDAL AWARD

June, 1954

DAVID GLENDENNING COGAN, M.D.
Boston, Massachusetts

Mr. Chairman, members of the Research Association, guests, and most particularly those members of the secret committee charged with the award of the Proctor Medal, I am deeply grateful. To one whose professional life is an attempted compromise between research, teaching, clinical practice, and a minimum of administration, this occasion is a happy milestone. The doubts which I have on the wisdom of your selection are momentarily overbalanced by the satisfaction that any of us might have when, standing in judgment before his colleagues and peers, he has been found not without merit.

But I would hasten to add that this supposed merit is a virtue dependent as much or more on chance as on inherent qualities. This award is in fact made to a congerie of circumstances which I happen to represent. These circumstances are too numerous, and many too personal, to enumerate fully, but I can't refrain from referring with gratitude to my predecessor in the Howe Laboratory, Dr. F. H. Verhoeff, whose uncompromising adherence to what he holds to be right has been my moral guide. Nor can I forbear mentioning my former associates, Dr. Elek Ludvigh and Dr. Everett Kinsey, and my several associates, especially Dr. Morton Grant, on whose counsel I have depended heavily over the years.

Awards such as this are said to have two possible effects on a person. They may make him grow in stature or they may make him merely swell. I pray that my lot may be the former for it is an exciting time in which to grow. Never before have the opportunities and facilities been available to a comparable extent for one who is seriously interested in pioneering in research. The only question is how to reconcile the demands of research with the material and emotional demands of modern living. To be sure this must be an individual matter, but it is perhaps the prerogative of one who has been through the mill, and made his full quota of mistakes, to offer a few observations.

In the first place I should like to set aside the popular misconception that research is a matter of test tubes and Geiger counters. The essence of investigation is originality, curiosity, and perseverance, and this applies to laboratory as well as to clinical research. Barriers separating the laboratory and clinical investigations have naturally widened with the acquisition of new knowledge and specialized techniques so that it is rare for any one individual now to encompass them both successfully. Thus has come about the development of the clinical research laboratory where the basic scientists and clinician may meet. I am proud to say that ophthalmology has been one of the leaders in the development of this type of laboratory. Made possi-

ble first through private beneficences and expanded more recently by government agencies, who can foretell what the future holds in store? All we can say at present is that this development has already had a profound effect on the teaching and research in this country.

The problem facing the individual, particularly the individual with academic aspirations, is how to acquire the broad training necessary for the general practice of ophthalmology and yet acquire the advanced knowledge of one small phase in which he hopes to make an original contribution. Many budding clinicians believe they can accomplish this by spending the spare time which they foresee in the early years of practice by working on some problem in a laboratory. It is my experience that most such high hopes come to grief. The attempt to learn research while conducting a practice usually leads simply to unhappy frustrations. Perhaps it would be otherwise in a clinic where one can schedule his responsibilities effectively but not where private practice is concerned.

On the other hand, if a person has learned the method of scientific enquiry and has identified himself in one field of research, however small, it is possible to pursue this with authority and savoir faire despite the demands of practice. Thus it seems to me of the utmost importance for those individuals with academic aspirations to spend one or more years in a laboratory devoting their full time to research and teaching and to pursue these with the same vigor and enthusiasm that they pursued their clinical traineeship. This sacrifice of one or more years in the early stages of one's training is small by comparison with the intellectual and humanitarian dividends which it is certain to yield in later life.

Finally, I would like to end by telling a story which has little bearing on the Proctor award other than that such an award gives rise to reflection on times past. It makes one speculate on how he would have done things differently. The story is that of a modern Faust who wanted to recapture his youth but instead of making a pact with Mephistopheles he obtained some hormones from his physician. He was instructed to take one pill a day but being an impatient sort he took the entire box full of pills the first day. The following morning his family had a time rousing him. After being shaken violently, he finally opened one eye and said "Alright, I'll get up but I won't go to school."

BIOGRAPHICAL DATA

David Glendenning Cogan, M.D.

At the time of this accounting in 1954, Dr. Cogan is the very active Director of the Howe Laboratory of Ophthalmology and Associate Professor of Ophthalmic Research, Harvard Medical School. He is also Chief of the Thursday Service and a Senior Surgeon of the Massachusetts Eye and Ear Infirmary, Director of the Eye Pathology Laboratory, and a member of the Editorial Board of the *A.M.A. Archives of Ophthalmology*. He serves as consultant to numerous national and international organizations, including the National Research Council, the World Health Organization, and the National Society for Prevention of Blindness, as well as the Los Alamos Medical Center, the U. S. Naval Hospital of Chelsea (Massachusetts) and the U.S.A.F. School of Aviation Medicine.

Despite these various commitments, Dr. Cogan's principal interest and activity is not in administering and advising but in actually carrying out experimental and clinical investigation. In his few nonophthalmologic

hours, Dr. Cogan easily metamorphoses into a gentleman farmer and woodsman. At his southern New Hampshire farm, with the help of his wife and four daughters, he attacks the practical problems of primitive living with the same enthusiasm and ingenuity as he approaches the problems of ophthalmology in the laboratory.

In arriving at his present status, Dr. Cogan may have been influenced in his youth by the example of his mother in her career as an ophthalmologist, and he may have been guided into righteous ways by the teachings of his father, a minister. Dr. Cogan was born at Fall River, Massachusetts, in 1908 and raised in this exemplary family background at Wakefield, Massachusetts. He graduated from Dartmouth College in 1929 and from Harvard Medical School in 1932. Following internship at the Chicago University Clinics 1932-33, and residency in ophthalmology at Massachusetts Eye and Ear Infirmary 1933-35, he made a brief excursion into the private practice of ophthalmology under the auspices of Dr. J. Herbert Waite, then Professor of Ophthalmology at Harvard. It soon became apparent to Dr. Cogan that the fascination of ophthalmology for him related to investigation rather than to application. He then joined his respected friend and mentor, Dr. F. H. Verhoeff, in the Howe Laboratory. From 1937 to 1938 Dr. Cogan studied in Switzerland, Germany, and Holland as a Moseley Traveling Fellow, accompanied and assisted by his wife, Dr. Frances C. Cogan, daughter of Dr. Joseph Capps, Professor of Medicine at the University of Chicago.

Dr. Cogan organized and established the Harvard Post-Graduate Course in Ophthalmology, and in 1940 succeeded Dr. Verhoeff as Director of the Howe Laboratory of Ophthalmology.

Dr. Cogan's best known works have been on the physiology and pathology of the cornea, on cataractogenesis by atomic radiation, and on the neurology of the ocular muscles. His list of publications reveals an even broader range of interests. Appreciation for his work has been attested to not only by the personal admiration of his colleagues but also by the presentation to him of the Warren Triennial Prize in 1943, the award of the New England Ophthalmological Society in 1953, and now the Proctor Medal of the Association for Research in Ophthalmology.

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A BRANCHED-PATHWAY SCHEMA FOR THE COLOR-VISION SYSTEM AND SOME OF THE EVIDENCE FOR IT*

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A seeming paradox in the workings of the visual system is the fact that the various types of cones make "equal" contributions to color, the while they make very unequal contributions to brightness.

One says that in a white illumination the physiologic substrates of the chromatic components (prosensations) aroused by the receptors are equal, in order to account for the fact that the sensation is achromatic. The assumption is necessary whether one adheres to the traditional view that whiteness is synthesized (from redness-plus-greenness-plus-blueness), or adopts the "excess hypothesis" according to which whiteness exists by default, when no prosensation exceeds another so that the imbalance can result in a corresponding huedness (see Walls, 1952).

On the other hand when working primary lights are mixed to match serially the segments of the spectrum, three curves of the intensities of the respective primaries versus wavelength are obtained. When these are manipulated to remove negative coefficients, by being transformed to hypothetic primaries having high saturations and the hues assumed for the prosensations, and are made to sum up to give the photopic luminosity curve, the areas under the three curves are very unequal. This is taken to mean that the respective receptor-types make unequal contributions to the brightness of a white light. The greenness receptor appears to make the largest contribution, the redness receptor a lesser one. The contribution from

* From the School of Optometry, University of California. Read, in condensed form, under the title: "Severe heterozygous actions of color-blindness genes," before the Association for Research in Ophthalmology, San Francisco, June 23, 1954.

the blueness cone is so small that the variations in the treatment of similar color-mixture data by individual investigators have sometimes made it appear to be zero or even weakly negative (implying an inhibitory action of the blueness receptor upon the brightness activities of the other two types).

Studies of data other than those of color mixture have led to proposals of even greater inequality of the brightness rôles of the color receptors. For a period, Arthur König even thought that all of the photopic brightness was given via the redness receptor. Some have even taken seriously, as physiologically meaningful, the arbitrary assignment of all brightness to the greenness receptor in the C.I.E.* system of colorimetry.

In every such schema, however, it will be found that any chromatic valence curves exhibited have equal integrals, equal areas beneath them, to preserve the three-component theory's explanation of "white" sensations (see for example Thomson and Wright, 1953). Assignment of all brightness to some one receptoral system is not, however, something to which only the three-component theory has ever been forced. The paradox emphasized in this introduction existed also for Hering, in whose original theory the red-green and yellow-blue receptors yielded chroma but no brightness—the latter all being given by a third, black-white, receptor.

This paradox has long seemed evidence for some degree of independence in the anatomicophysiologic mediation of photopic brightness and of chromaticness (that is, color per se, a hue at a saturation). It is, however, only a part of the evidence for somewhat separate, somehow separate, brightness and chromaticness mechanisms.

The "flicker" photometer is an interesting instrument which operates on a principle which everyone can expound and no one can really explain. If two illuminations of different color and unequal intensity are alternated in a single area at an increasing rate, their colors combine into a unitary steady color at a relatively low frequency, with their brightnesses still unfused and causing "flicker." This is eliminated by adjusting the intensity of one of the lights without any further increase of the alternation-rate, whereupon the two intensities can be pronounced photometrically equal. It goes overlooked by the photometrist (since the procedure would defeat the purpose for which he uses the instrument) that if the original intensities were left unchanged, the brightness flicker which remains after the chromaticnesses have fused could be eliminated by raising the alternation-rate to a critical frequency. For chromatic flicker to be eliminated at one frequency and for brightness flicker to "fuse" only at some higher frequency is prime evidence for an independence of physiologic mediation.

The binocular integration of chromaticnesses also behaves differently from the combination of brightnesses. The latter, in themselves, never "rival" (though the lightnesses of surface colors, which are brightnesses relative to surround brightnesses, can do so). In binocular rivalry of forms, there is usually either a mosaic aspect or, if the forms are simple, all-or-none replacement of one eye's material by the other's. The colors of haploscopic areas instead rival by "wiping each other off" of the area for which they are contending. When the hues and saturations of heterochromatic half-images are such that they cannot be fused, it will always be found that any textural landmarks on both monocular stimulus-areas are all visible continuously during the rivalry of the colors, showing that the brightness aspects of the half-images are being held combined in a stable manner. When the members of a stereo-pair of configurations are of two unfusible colors, it will be found that stereo-

* C.I.E. abbreviates Commission International d'Éclairage, the international standardizing body for photometry, colorimetry, and radiometry.

scopic depth and tridimensionality is seen continuously even while only one of the colors or the other monopolizes consciousness.

The investigator who has been most interested for the longest time in the puzzle of the independence of brightness and chromaticness is, undoubtedly, Henri Piéron. One may suppose that he has put into his recent book (1952) all of the evidence he can find for independence, for he would wish to put in the best light the solution he himself offers for the puzzle. In the book, I have been able to find the following points made: Individual requirements of the intensities of the red, green, and yellow lights for a color-match in an anomaloscope do not correlate with the individuals' luminosity curves. Anomaloscopic mean intensities do not correlate with purity limens for the wavelengths used in the instrument. Mean anomaloscopic red-green ratios do not correlate with the individual observer's spectral locus of "purest yellow." "Chromatic fatigue" is much more rapid than brightness-wise adaptation. A subject may have a normal luminosity curve and abnormal color-mixture data in spectrum gauging, while another subject may have orthodox color-mixture data and an abnormal luminosity curve. The slightest of cortical lesions may impair or abolish color vision leaving intensitive vision normal; and, during recovery from an injury which has caused temporary hemianopia, there may be a hemiachromatopsia.

In résumé, the things which Piéron has found himself and quotes from others, together with the independence of chromaticness and brightness in binocular fusion and rivalry, the distinctness of their critical flicker fusion frequencies, and above all their differing quantitative constitution from the offerings of the retinal receptor-types, make an impressive case for separate mediation.*

In turn the most impressive hypothesis regarding a mechanism for the separate mediation is that of Henri Piéron's receptoral tetrad, which is most fully expounded in his book. The proposal in brief is that there are three "chromatic" kinds of cones, each with its own photochemical substance, hence having a uniquely shaped absorption-spectrum curve. Each contributes a prosensational huedness to sensation—redness, greenness, or blueness—but, no brightness. All photopic brightness is given by a fourth "achromatic" type of cone. This contains all three photochemical substances, but in an imbalance of relative concentrations which gives the absorption curve of the cone-as-a-whole the shape of the photopic luminosity curve of the eye.

Piéron has had to complicate this simple idea immensely, with a maze of hypothetical interconnecting retinal circuits which enable various receptors to exert inhibitions in various ways, in order to make his system appear workable. But there is a fundamental objection to be made to the simple idea itself. This is, that it resolves one paradox only to create another. The thresholds found in the various kinds of visual discrimination which are all only special cases of intensity discrimination—the kinds of visual acuity we call resolution, vernier acuity, the angular acuity of visible motion, stereo-acuity—are entirely too small to account for if only about one out of every four of our retinal cones can contribute to brightness and can share in spatial vision. On the other hand the data of the areal thresholds for chroma are too fine to explain if only something like three-fourths of the cones contribute to color.

There is more than a little similarity to Piéron's idea of separate brightness cones in Granit's (1947) dominator-modulator hypothesis of the organization of the retina. While I have elsewhere explained why Granit's modulators need not have anything

* And emphasize the unwisdom of official international colorimetry in insisting that brightness is a dimension of color itself, making photometry a mere branch of colorimetry.

to do with color vision (see Walls, 1953), let us here allow Granit to say what they do. Each modulator system of the retina is an optic-nerve fiber into which funnel receptors of a single type, having a narrow and specifically located spectral absorption curve. A given type of modulator arouses a specifically hued component of sensation—but no brightness. All brightness is aroused by dominators. A photopic dominator system also consists of an optic nerve fiber and all the retinal elements connected with it, but with the receptors concerned representing a full set of all of the types present in isolation in the various modulators. This makes it possible for the dominator mechanism to respond throughout the spectrum, in quantitative accordance with the ordinates of the photopic luminosity curve, just as Piéron's photochemically "mixed," purely brightness-giving cone is able to do in a far simpler way.

The same objection, stemming from the embarrassing facts of the visual acuities, and so forth, applies to Granit's schema as to Piéron's.* The dilemma upon which both of these most modern hypotheses are impaled, together with all cruder ideas offered in the past along similar lines and now of only historical interest, can be avoided only by a proposal which allows each type of cone to make a contribution to chromaticness and also to make a contribution to brightness, with the redness, greenness, and blueness cones contributing to color per se in one triple ratio (say 1:1:1) but simultaneously making contributions to brightness in a totally different triple ratio (say, 4:5:1).

Just such a proposal was made, in words only, by Walls and Mathews in 1952. I am now prepared to amplify it and to illustrate it with diagrams. The evidence for any theory consists of the facts it is capable of explaining. In the case of the branched-pathway hypothesis, most of the facts relate to the color-blindnesses. I am one of those who, convinced that each kind of hereditary, congenitally defective color-vision system represents a normal system minus something (or -things), believe in allowing the color-blindnesses to mold a schema for the normal into which they will, necessarily, fit. This point of view has a long history tracing back before Helmholtz to the earliest work of James Clerk Maxwell, and it is every bit as respectable, scientifically, as the thesis that one can work out the details of the normal system by studying only normal subjects. The branched-pathway theory, it will be seen, owes a special debt to a handful of cases of seriously defective color vision in persons who "theoretically" should be normal—they are women who have only one gene for any one kind of color blindness. The interpretation of the first of such cases seen in our laboratory was what forced Walls and Mathews (1952) to the conception of branched pathways in the first place. No case seen since has failed to be capable of being fitted into the schema.

The schema in its present form is exhibited in Figure 1. This is not an anatomic diagram, but a physiologic one. The brightness and chromaticness stations are not "centers" but populations of elements which may be in the retina or may be in the cortex. The pathways shown traveling upward from the receptors are not fixed in size. Most would expand and contract variously with various chromatic illuminations of the receptors, since they are streams of nerve impulses. In particular the impulse flux arousing brightness from rod activity would be huge in scotopia.

The three color-cone types are considered to contain respective photochemicals L, M, and S. These have their absorption peaks in

* Moreover the dominator-modulator mechanism is quite unworkable for the rod-free area of the fovea. There is no histologic funneling there which could represent the combination of several modulator receptors into one dominator system—and yet, photopic brightness is certainly not lacking. Piéron's receptoral-tetrad schema can avoid this same sort of difficulty only if he lifts its switchboard of cross-connections entirely out of the retina and places it in the cortex. Foveal cones, their bipolars, and their ganglion cells simply do not have any such intraretinal cross connections.

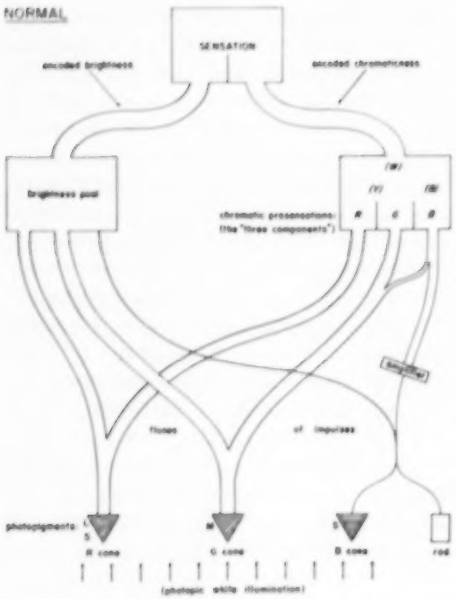

Fig. 1 (Walls). Schema for normal color system.

the longwave, middle, and shortwave moieties of the spectrum. The **R** cone, responsible for the *R* prosensational process which introduces redness into the sensation when allowed to (that is, when it is not the least prosensation or the only one present), normally contains a little S. This causes the familiar secondary shortwave peak on the curve of the distribution of *R* along an equal-energy or a white-light spectrum. The capacity of **R** to synthesize this S is precarious, for it promptly disappears nearly or quite completely in even mild protanomaly, in deuteranopia, and in tritanopia. The **R** cone is thus somewhat abnormal in almost all kinds of color blindness and not only in the protanoid defects.

The sharing of conducting pathways by rods and (exclusively or at least largely) **B** cones explains the bluishness of scotopic "white": a few impulses from the then strongly active rods can leak up the blue-huedness pathway, and these are somehow not wholly kept from chromatizing the sensation by the "excess" mechanism.* Such sharing is logical enough. If some cones are to transmit over some of the same fibers that carry rod messages, it is the **B** type which can best be doomed thus to diffuse connections at various levels; for, playing no material rôle in intensitive vision, the **B** cones have no responsibilities with regard to the faithful point-for-point transference of the optical retinal image to the cortex and consciousness. Only an optic-nerve fiber, and never a single receptor out of many connected therewith, can have a "local sign."

The main bifurcations of the impulse-fluxes may well occur within the retina itself, although the evidence for this does not yet seem overwhelming. In the interest of economizing on conductors, it would seem better for the system to accomplish the bifurcations as close to the cortex as possible, or within it. On the other hand, some rare color-visual defects that must involve the pathways rather than the receptors can occur monocularly but have not been found to occur hemianopically—as though most of the features of Figure 1 were retinal as to locus. And, the selfsame optic-nerve fiber might very well carry both encoded brightness and encoded chromaticness messages. The on-effect in an on-off fiber might connote brightness while the off-effect (already known to be subdivided; see Walls, 1953) may signal hue-and-saturation.

I assume that the relative fluxes entering the "brightness pool" are quite directly determined by the photochemical concentrations in the receptors. The assumption is bolstered by independent evidence that the concentration of S in **B** cones is far lower even at maximum than the concentration of **M** or **L** (Walls and Heath, 1954). Individual differences in the luminosity curves of normals would thus primarily express

* Ordinarily, when only one prosensation is activated, there is no hue in the sensation since there is no other prosensation for the given one to exceed (Walls, 1952; Walls and Mathews, 1952; Walls and Heath, 1954).

small differences in the relative concentrations of L and M, as may local differences in the retina of the same individual.

The relative fluxes starting toward the chromaticness blending station can be assumed to be in the same triple ratio as the three brightness messages.* The chromatic branch from **B** must, then, pass through an amplifier however, in order to bring B up to par with R and G. There may be amplification also in the R pathway (since only the largest, G, would certainly not need any); but I have taken the liberty of suggesting that the diversion of some potential G flux to the production of a little B by the **G** cone cuts down the definitive G to neat equality with R and B in the neutral, white-light situation.

This twig from the **G** cone to the B compartment seems necessary to assume for several reasons. Without it, it is very difficult to explain the low saturation of spectral yellow, since the consensus is that the **B** cone has no responsivity to wavelengths longer than the yellow-green. The twig makes unnecessary the desperate resort of no less than three sets of investigators, who have independently suggested that R can desaturate G and vice versa when only these two prosensations are activated, with both R and G nevertheless entering into the hue and making it yellow or at least yellowish (see Walls and Mathews, 1952). The slight yellowishness of even extreme longwave spectral red is similarly accounted for, for while under the excess hypothesis the hue there should be that based upon R itself if only R and G are present with R in excess, the G can influence the hue (by entering with some R into the synthesis of some Y) if there is even a little B "beneath" it.

When it is understood—from the G-to-B twig—that the total B valence curve is compounded, from a large "B_b" contribution from the **B** cone and a small "B_g" aroused by the **G** cone (and proportional, at any wavelength, to G itself), it is seen that the chromatic contribution from the **G** cone is as if the greenness sensory component of colors were not a pure green but a somewhat bluish green, a bG and not a G. This seems to explain why in acquired "tritanopia," as may occur with retinitis or detachment, the knocking out of the **B** cone and of most or all S-synthesis does not eliminate all bluishness from sensation: the average victim of acquired "tritanopia" finds that the one hue remaining to him at the short-wave side of his yellow neutral point is a blue-green.

Lastly, the G-to-B twig explains why a deuteranope's neutral-point wavelength is so close to a protanope's. If deuteranopia is indeed a "collapse" dichromasy in which the **R** and **G** cones both contain L and no S, the deuteranope's B_g curve will lie redward from the normal's since it is shaped by the properties of L and not M; so, the total B curve will lie lower where it intersects R and the intersection will not lie far from where B and G intersect in the protanope. In my special pseudo-deuteranope, V.P. (to be reported herein), the lack of any B_g moves her neutral point even farther violetward, so that it lies in the no-man's-land between the lowest value for any deuteranope (out of more than 50 studied so far) and the highest value yet found for any protanope (among, again, more than 50).

The modifications of the normal schema necessary to depict the standard dichromasies are given in Figures 2 to 4. Each is quite in line with orthodox suppositions as regards the underlying bases of these conditions. Under three-component theory, they are each readily interpretable as having the basic defect at the receptoral level.

The protanope (fig. 2) lacks **R** cones completely, hence suffers a substantial loss

* But they need not be. If each bifurcation occurs in a synaptic bed, there is no reason why the branches beyond the bifurcation must be equally large. Relative "amplification," or its antithesis, of either branch could be brought about by multiplication or minification of paths in the reciprocal-overlap organization of any synaptic bed.

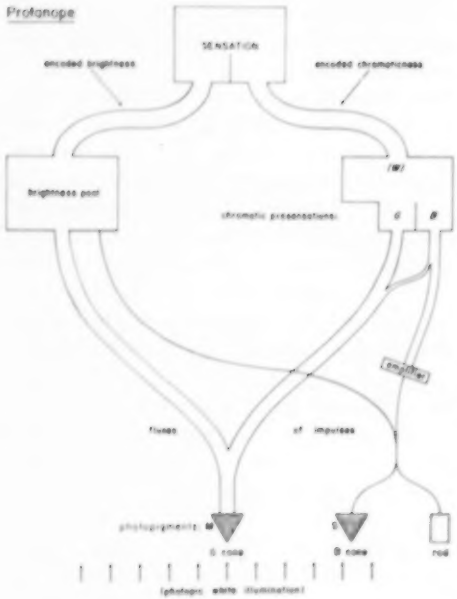

Fig. 2 (Walls). Schema for a protanope.

of brightness (which is more and more conspicuous as wavelength increases), and cannot experience redness. His only hues are those relating to *G* and *B*, separated by the neutral point where neither *G* nor *B* can be in excess, and with each prosensation serving only as a desaturant in the half-spectrum where it is the lesser one.

The deuteranope (fig. 3) has the magnitudes of *R* and *G* forever "equal" (or at least in a fixed ratio) since **R** and **G** both contain only L (very likely in equal concentrations since his luminosity curve is claimed to peak a little redward, on the average, from the normal's). His only hues are yellow and prosensational blue, with spectral saturations determined just as they are in the protanope.

Congenital tritanopia appears to have been proved to be, like protanopia, a "loss" system (Wright, 1952; Thomson and Wright, 1953). With no **B** cones and no B pathway or other mechanism (fig. 4), the only possible hues are prosensational red and green, with each prosensation capable of desaturating the other when it is the lesser, and with a neutral point where the two are equal.* The luminosity curve should be about as little abnormal as a deuteranope's, since the loss of the **B** cones should not alter it materially. The probable situation in acquired "tritanopia" is shown in Figure 5.

While the common dichromasies and the normal trichromatic system can thus all be represented by branched-pathway diagrams

* The congenital tritanope's neutral point is at a wavelength about 10mμ shorter than that at which the normal finds his purest yellow. If the tritanope's **R** cone contains extra L replacing the missing S, the *R* valence curve will be elevated and its intersection with the *G* curve (determining neutral-point position) will be displaced a bit violetward into the (normal's) yellow-green region.

There are indications, to be seen in the work of Wright (1952), that the congenital tritanope really has a second neutral point very near the violet end of the spectrum. This would mean that his **R** cone is not entirely devoid of S, and that the shortwave hump of the *R* curve is still present although very low, and manages to intersect the *G* curve before both reach the baseline at the commencement of the ultraviolet.

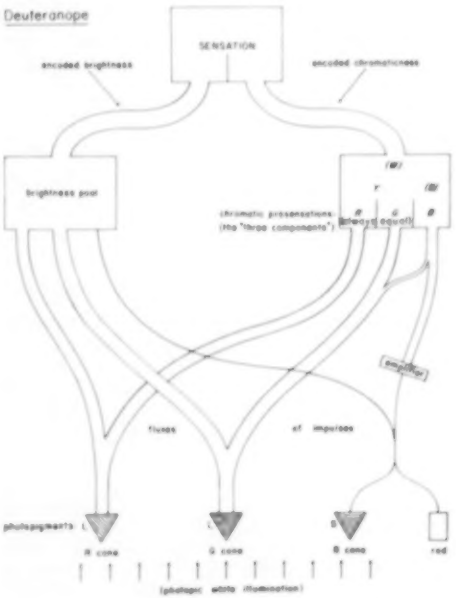

Fig. 3 (Walls). Schema for a deuteranope.

which are perfectly compatible inter se, I wish to reiterate that the whole branched-pathway idea arose from studies of uncommon conditions, namely the unexpected states of affairs that can occur in females heterozygous for sex-linked color-blindness genes. It is to these that we now turn.

It is well known that many of the genes in the human X chromosome are concerned in one way or another with responsivity to light, and that most or all of these genes have some penetrance so that the normality allele of each is imperfectly dominant. The heterozygote for protanopia, protanomaly, deuteranopia, or deuteranomaly is perhaps never perfectly normal. Such "carriers," at least statistically, have elevated purity limens, widened ranges of anomaloscopic matching, and so forth (see Walls and Mathews, 1952). It is not at all possible to identify clinically every individual carrier, hence it is not possible to assure every seemingly normal girl that she cannot have color-blind sons.

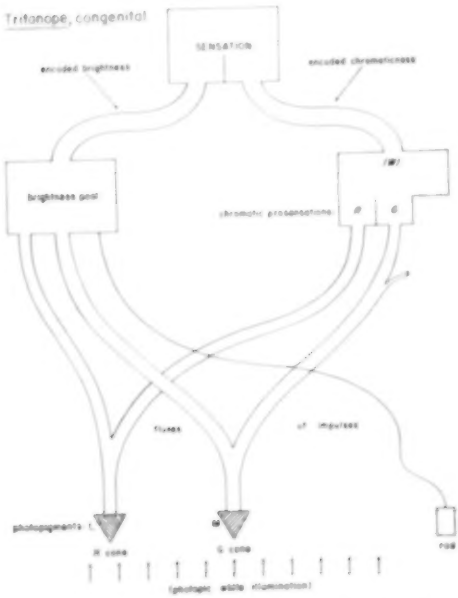

Fig. 4 (Walls). Schema for a congenital tritanope.

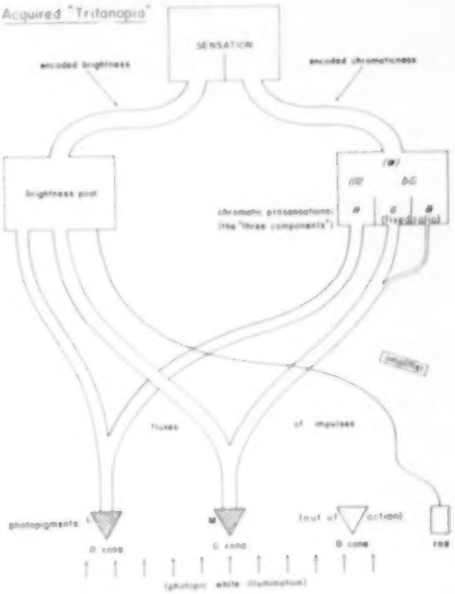

Fig. 5 (Walls). Schema for an "acquired" tritanope.

An occasional female who cannot be homozygous for any color-vision defect is nevertheless seriously color blind. Such a female, if her father is color-normal, can only be heterozygous—obtaining her one deficiency gene from an also heterozygous mother who is phenotypically normal. There should be an equal number of seriously defective, but merely heterozygous, daughters of defective fathers and homozygous-normal mothers. Such a female would usually be hard to identify as a heterozygote if one had to try to prove that her mother could not be a heterozygote.

Since the original discovery of these "manifesting heterozygotes" for deuteranopia, protanomaly, and so forth, it has been universally assumed that each one is truly manifesting the very defect for which she is heterozygous. Results with pseudisochromatic chart tests, or even with an anomaloscope, would not lead the average investigator to suspect anything else. It also seems to

be generally assumed that heterozygotes for any given defect must form a continuum, from a large majority who cannot be found to be even slightly abnormal by any available test down to a tiny number who show the defect in full bloom as if they were homozygous for it. I suspect that both of these generalizations are completely wrong.

In every hundred men, on the average, there are five deuteranomals, a deuteranope, a protanomal, and a protanope. Since a female has two X chromosomes where a male has only one X and a Y, the opportunity is twice as great for a female to have an X-linked defect-gene as for a male to have one. Eight percent of males are color blind in the common ways; but an amazing 16 percent of females—one woman in six—are carriers. The clinical normality of nearly all of these is obvious to anyone.

Of the 16 carriers among every 100 women, 12 carry deuteranoid defects. None of these, as an individual, can be identified at present as a carrier from anything about her own vision (unless she is the rara avis who seems to be deuteranopic or perhaps deuteranomalous). With the one carrier in four who is heterozygous for a protanoid defect, the situation is different. Prot-heterozygotes do not form a continuum between normality and "protanopia" in respect of their color vision, but a large majority are easily identified by their brightness vision.

When one has a male protanope in hand, one knows that his mother must have at least one gene for protanopia—he could not have gotten one from his father and still have been male himself. If the mother is living and available for testing, the chances are ponderous that nothing wrong will be found with her color vision: she will pass the Ishihara or the American Optical chart test without error, will set the anomaloscope within normal limits, will describe a normal RDP* (Walls and Mathews, 1952), and will have no family reputation for errors in color identification and matching.

But if a few points are obtained on her photopic luminosity curve it will probably be found that she has a depression of long-wave brightness. As a rare extreme her luminosity curve may be exactly a protanopic one instead of "halfway" between this and a normal one.

This phenomenon has been called for short, after its discoverer, "Schmidt's sign" (by Walls and Mathews, 1952). The underlying situation is depicted in Figure 6. In the prot-heterozygote who reveals herself by showing Schmidt's sign, the redness receptors are not lacking as they are in a protanope, nor reduced in numbers and photochemically abnormal as in a protanomal. They are normal, and normal in numbers, which is why the Schmidt's-sign female is not a protanomal although she seems to have the luminosity curve of one. Rather, the defect she does have is at the pathway level.

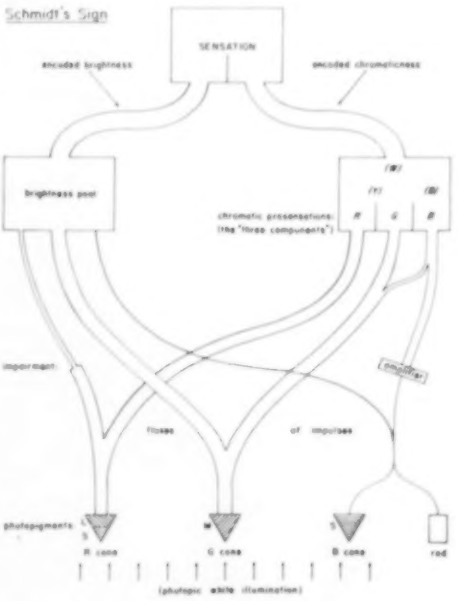

Fig. 6 (Walls). Schema to illustrate the phenomenon of Schmidt's sign.

* Receptor-type distribution pattern.

The brightness branch of the **R**-cone path is somewhere, somehow, impaired so that it carries a reduced flux of impulses. Nothing else is wrong—but this is enough to dim for such a woman any yellow light, more so an orange, and particularly a red light, while leaving her color vision completely undisturbed.

The first—to my knowledge—"completely manifesting" heterozygote for a sex-linked dichromasy to be identified in this country was B. L., first described by Walls and Mathews (1942; as Ba. L.). She is a dichromate with a color-normal father, but for her the question of whether her putative father is her real one or not does not even arise. For, B. L. is one of a pair of monozygotic twins and her otherwise identical sister is color-normal. Both of the girls and their mother show Schmidt's sign and one maternal uncle is a perfect protanope (fig. 7). There can be no doubt that B. L. bears one gene for protanopia and no other color-defect gene.

B. L. suffers no more longwave depression of her luminosity curve than does her sister or the mother. If she were a genuine protanope she would lack **R** cones and would then exhibit a protanope's luminosity curve. Her **R** cones instead are as normal in numbers as her sister's or her mother's. But, though she obtains some brightness from their activity (as much as her mother and her color-normal sister do), she obtains no redness. The situation must be as shown in Figure 8. The receptor layer is normal but one entire color-component has been

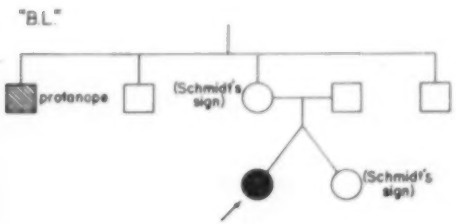

Fig. 7 (Walls). Pedigree of B. L.

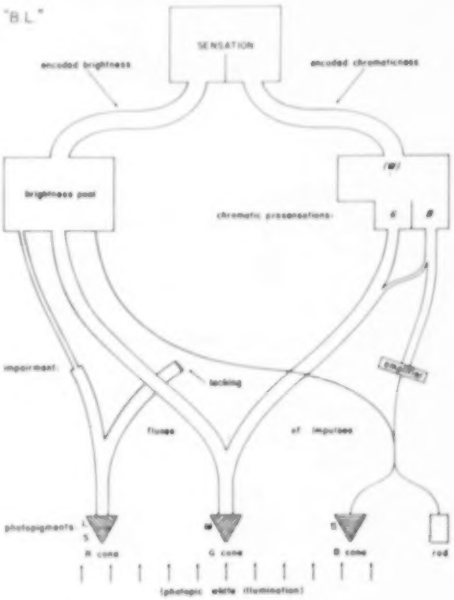

Fig. 8 (Walls). Schema to illustrate B. L.'s color vision

knocked out "centrally" through failure of a "redness" branch to develop at all. B. L. consequently has the color vision (but not the brightness vision) of a protanope, with the same two and only two hues in her spectrum. Her neutral-point wavelength is precisely at the mean protanopic value: $\lambda 491.2 m\mu$ (with respect to a C.I.E. Illuminant C "white").

The situation in Schmidt's-sign protheterozygotes in general and B. L. in particular convinced Walls and Mathews of a branching of the path upward from at least the **R** cone. Similar bifurcations of conduction from **G** and **B** cones remained mere assumptions at that time. Shortly, however, V. P. came to light.

I learned of V. P. through a former college teacher of hers who had confirmed her flagrant color-defectiveness with an Ishihara test and had lent her the book with which to test her family during a vacation period. She reported back that her brother, sister,

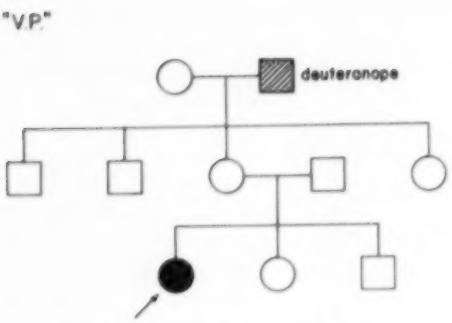

Fig. 9 (Walls). Pedigree of V. P.

mother, and father were all normal (fig. 9). This seemed impossible unless V. P. were a "manifesting" heterozygote for some sex-linked defect, or else homozygous for some autosomal and recessive type of color blindness. Yet V. P. was not an achromate, nor was there any suspicion that she was a tritanope.

I was able to study the immediate family with the methods of Walls and Mathews. V. P. simulated deuteranopia—she was dichromatic but had a clinically normal luminosity curve. But two things made her different from any known genuine deuteranope: her neutral point lay between the deuteranopic and protanopic wavelength bands (see above), and she described an RDP of normal configuration and used the word "red" for its color, whereas a true deuteranope sees no such RDP (see Walls and Mathews, 1952). These points were enough to make it seem unnecessary to question her mother's morals.

V. P.'s mother is inescapably a heterozygote for deuteranopia, for this woman's father, when eventually tested, proved to be a perfect deuteranope with no RDP and with an orthodox neutral point (at λ496.1mμ).

The mother could have had no additional gene for some other defect in her other X chromosome, to give to V. P. instead, or her son would have received that gene and would not be color-normal as in fact he is. V. P., then, is a simple heterozygote for deuteranopia. Her RDP being normal, there is no reason to suppose that her retinal receptor layer is in any way abnormal, let alone abnormal in the way a real deuteranope's is (fig. 3; cf. fig. 1).

On the other hand, with the "precedent" set by B. L. in mind, it is easy indeed to account for V. P.'s visual peculiarities by, again, a pathway defect: she has simply never developed the greenness branch of her G-cone path (fig. 10). This renders her dichromatic, but should make the two hues in her subjective spectrum blue and red whereas those of a genuine deuteranope are known to be blue and yellow (from "unilateral" cases, where one eye has near-normal color vision).

Neither B. L. nor V. P., then, actually does manifest phenically the defect for which she harbors one gene. Essentially this is because when one defect-gene in a female

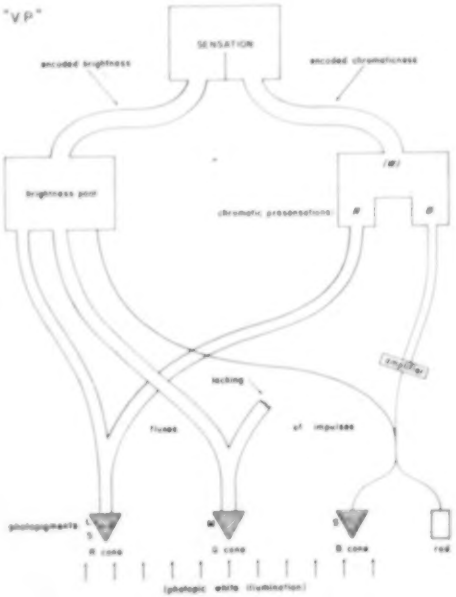

Fig. 10 (Walls). Schema to illustrate V. P.'s color vision.

does have a severe action, it attacks the pathways of the color system and not the receptors—where that same gene, in a male, or doubled in a homozygous female, would operate. Such a difference in site of action appears to be without precedent in the physiologic genetics of any known animal organism. And, it is clear that without the branched-pathway schema here hypothesized for the normal, it would be difficult to explain the sparing of color and the reduction of brightness in Schmidt's sign, or the selective destruction of a huedness with sparing of the associated brightness in V. P. or in B. L.

No exact tritanoid counterpart of B. L. or V. P. has been seen. But, "impairment" of **B**-cone brightness would hardly be detectible, and absence of the blueness pathway would be indistinguishable from homozygous tritanopia. We shall shortly see, however, that "manifesting" heterozygotes for tritanopia may be identifiable all the same—they may be what the scientific world has been calling tetartanopes.

It appears proved that the gene for protanopia and its normality allele are interchangeable at one locus in the X chromosome, with the deuteranopia gene and *its* normality gene interchangeable at a separate and distinct (though not distant) locus in the chromosome (see Walls and Mathews, 1952). It is consequently possible for a woman to be heterozygous for both protanopia and deuteranopia at once, having a gene for one defect in one X chromosome and a gene for the other in the other X—or, even, both defective genes in one X and normality alleles at both loci in the other.

Any such "compound heterozygote" should be color-normal (if either defect-gene, present in her alone, would leave her normal). Only four such double carriers of protanopia and deuteranopia have ever been described. The first was identified by her having produced both protanopic and deuteranopic sons. She herself was either normal or at most only mildly anomalous (Göthlin, 1916, 1924). Another, studied by Francheschetti (1949) and found to be perfectly color-normal, was the daughter of respectively protanopic and deuteranopic parents and hence inevitably a compound heterozygote. A third, diagnosed through the fact that her only children (two sons) were compound hemizygotes—with color systems combining protanopic and deuteranopic effects—was described by Walls and Mathews (1952); she was color-normal by every test except for a deuteranomaloid mean setting on the anomaloscope.

Interest here centers upon the fourth case, who, instead of being normal, was totally color blind. This was a woman designated "F2" by Hylkema (1942). He did not understand her status, but thought her to be achromatic because she was a compound heterozygote. She had a protanopic son and a deuteranopic son. Her visual acuity and her brightness vision (as evidenced by critical flicker-fusion frequencies) were normal. She was not at all a "typical achromate" (see Walls and Heath, 1954) of the sort who has 20/200 vision, photophobia, nystagmus, and a dystrophic macula. I make no doubt that she would have exhibited a Purkinje phenomenon and a normal (or Schmidt's-sign) photopic luminosity curve, if Hylkema had looked for these features.

F2 is readily interpreted, and readily fitted into the branched-pathway schema for the normal (into which, if it is correct, all congenitally defective systems must be capable of fitting), if it only be assumed that F2 is, so to say, a combination of B. L. and V. P. If both of her two different defect-genes have exerted the severe, pathway-level action that each has had in one of those two girls, then her system is as shown in Figure 11. Reduced to a single chromatic prosensation, and with this (under the excess hypothesis) incapable of colorizing her photopic sensations, she is necessarily an "atypical achromate."

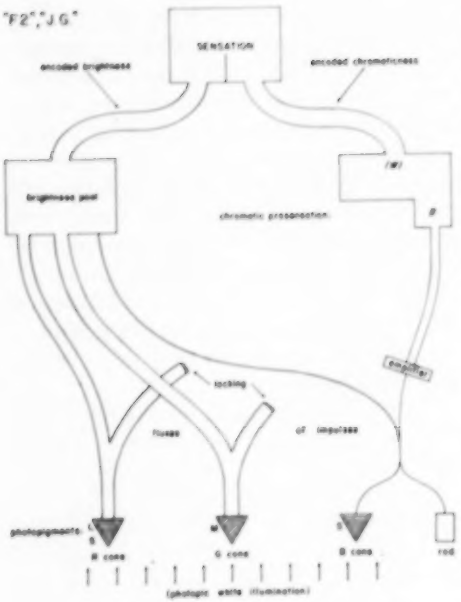

Fig. 11 (Walls). Schema to illustrate color vision of F2, J. G.

It does not seem to be essential for a color-blindness gene to be one for a dichromasy, in order for it to be capable of eliminating a color-component completely in a heterozygote as a rare phenomenon. Recently Weale (1953) published visual data for three "atypical" (normal acuity) achromates. Two were males and might each be explained variously, since for example the simultaneous inheritance of either protanopia and tritanopia, or deuteranopia and tritanopia, should render the individual achromatic but leave his visual acuity normal or even a bit supernormal. Weale's third subject was a woman, J. G., whose visual data set her apart in three or four respects from the two males.

Fortunately Weale gave enough family data so that it can be seen that J. G. must be a compound heterozygote (fig. 12). With an extreme-protanomalous father, she could not but receive from him an X chromosome containing a gene for extreme protanomaly (which is allelic to protanopia and ordinary protanomalous trichromasy). But she could not have had a deuteranomalous son unless she has in her other X chromosome (received from her mother) a gene for deuteranomaly (allelic to deuteranopia). The fact that her husband is color blind in some undiagnosed way has nothing to do with the interpretation of J. G. through her father and her son—in fact, it would not matter who her son's biological father was.

For J. G. to be achromatic must mean that effects just as severe as those F2 suffers can be exerted even by solitary genes for the sex-linked anomalous trichromasies. I could not draw separate diagrams for J. G. and F2; Figure 11 suffices for both. The lessons to be drawn from B. L., V. P., F2, and J. G. are that things in the realm of color blindness are not always what they seem—and, that "manifesting" heterozygotes henceforth must not be assumed to be showing what they carry until they are proved to be doing so by means of an extensive battery of tests.

The rarest of all the kinds of congenital color blindness is "tetartanopia." In the world literature I can find only an even dozen cases which, under the most liberal of definitions, were tetartanopes. Scarcely any two have been exactly alike as regards even the incomplete data ordinarily available. For the case or two ever exhaustively described, there are no exact counterparts. It is this

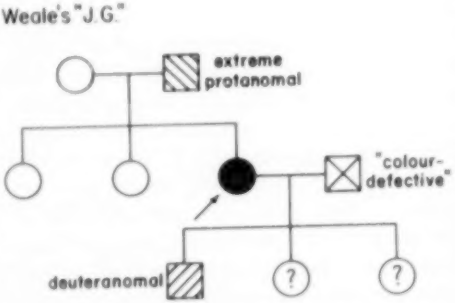

Fig. 12 (Walls). Pedigree of Weale's J. G.

individual variability of tetartanopia that I would stress first and foremost.

In a "book" case of the condition, the individual finds two neutral points in the spectrum, in the yellow region and in the blue. Between these he sees only one hue quality, presumably the normal's *G* hue. The two end-regions beyond the neutral points are both red and wavelengths from both can be matched to each other by equating brightnesses.

Color-wise, the situation is exactly what would be predicted under the modern three-component theory if the **B** cone were to be missing as in hereditary tritanopia, but with the **R** cone remaining normal. The only prosensational valence curves then present, *R* and *G*, would intersect twice to produce the neutral points; and, with *R* above *G* in both end-regions the only hue possible in either would be red.

If the whole situation were this simple and had this basis, tetartanopes would be as much alike as protanopes are. But perhaps the most conspicuous thing that tetartanopes do have in common is a great and unpredictable departure from normal spectral luminosity distribution. For only about half of the cases were any dependable luminosity data ever obtained. In at least four of these the curves were characterized by two widely separated peaks, or one very abnormally located peak, or a greatly enlarged area, and so forth. It is in respect of their brightness vision that tetartanopes differ the most; and it is just this that cannot be explained by the absence of **B** receptors and an otherwise more nearly normal system than that of a tritanope.

I shall offer here only an analysis of the one full description most comprehensible to me—that by Richardson-Robinson (1923), of the only tetartanope ever to be described from the Western Hemisphere, Mr. D. I have corresponded with him and I believe he is still living; but I have not been able to study him myself. Richardson-Robinson's work with him actually leaves nothing to be desired excepting the obtaining of his entire photopic luminosity curve referred to an equal-energy spectrum, which she never did secure.

Color-wise Mr. D. is a diagrammatic case, reporting only red at both ends of the spectrum and green in the middle, between neutral points in the expected places. But the brightest point in the spectrum of any continuous source was always in the blue or violet. The major peak, if not the only one, in his luminosity curve is at about λ466mμ. Despite his inability to experience blueness, it is inconceivable that he lacks **B** cones since his photopic luminosity curve is essentially the absorption spectrum of a **B** cone, summed with greatly reduced curves for **R** and/or **G** cones. Most normals find the purest spectral blue at about λ470mμ.

While blue objects have no blueness for Mr. D., they have an intensitive property which would not be predictable from his luminosity curve alone. They are often brighter and whiter than white objects. This seems physically impossible when one remembers that no blue surface could ever reflect more of the energy in the blue wavelength-band than a high-reflectance white surface does. Nevertheless when one rich blue paper was laid alongside a white paper before Mr. D., he called the blue one white and said that the white paper was dingy by comparison. On another occasion when the investigator had written him a note with blue ink on white paper, Mr. D. saw no writing on it until he caught the light on the paper from an angle which showed up the marks made by the pen. For Mr. D., the particular ink and the particular paper were a brightness match. By contrast a protanope, who cannot see redness in red-ink writing, sees the writing very plainly indeed—as black writing. Mixing the inks in their bottles is an age-old trick played upon protanopic bookkeepers!

No possible defect at the receptoral level could explain Mr. D. If we assume instead

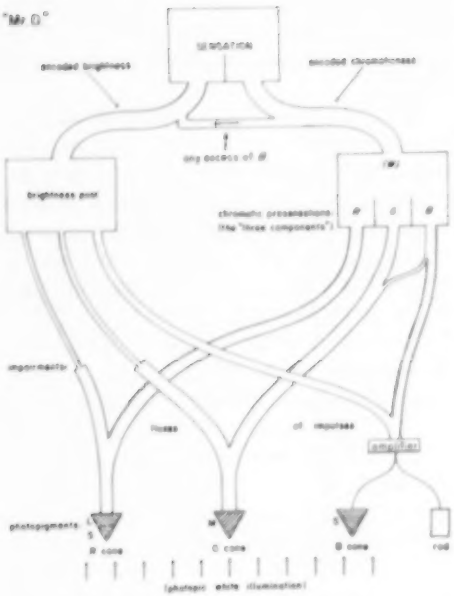

Fig. 13 (Walls). Schema to illustrate color vision of Mr. D.

that he has normal receptors and abnormal pathways, his system may be as shown in Figure 13. The amplifier is mislocated in the **B**-cone system so that the **B** cone makes much the largest contribution to brightness. Impairments like that which causes Schmidt's sign exist in both the **R**- and **G**-cone systems. The absorption spectrum of the S in the **B** cones is therefore the chief determinant of the photopic luminosity curve and accounts for its highly abnormal maximum in the blue region, together with normal spectral limits. What makes Mr. D. a tetartanope, however—a "blue-blind" or "blue-yellow-blind" dichromate—is the fact that any chromaticness flux that ought to be arousing a blueness hue component in sensation is somehow giving him instead extra brightness. The diversion cannot occur between the **B** cone and the brightness pool, else a white stimulus would be brightened as well as a blue one. For Mr. D. to see some blues as brighter than whites, the diversion of chromaticness flux so that it has a brightness effect must occur, as shown in Figure 13, high in the system.

Just such defects may never have developed in any other individual. The point is, however, that Mr. D. is a tetartanope and that his vision can be explained entirely at pathway levels without assuming anything but absolute normality in the receptor layer of his retina. This, be it noted, he shares with B. L., V. P., F2, and J. G.

Those women are heterozygotes for sex-linked dichromasies and anomalous trichromasies. If color-blindness genes of all sorts have their heterozygous actions (when they have any) on the branched pathways instead of on the receptors, then Mr. D. and any other tetartanope is under suspicion of being a heterozygote. A male cannot be heterozygous for protanopia, protanomaly, deuteranopia, or deuteranomaly. But he can be heterozygous for tritanopia, for in Wright's abundant material it can be plainly seen that tritanopia is inherited like any simple recessive trait. Its gene locus is either in an autosome, or else in the homologous segments of the X and Y chromosomes. Indeed, all known tetartanopes but one have been males (their number is too small for a 1:1 sex ratio to be a reasonable expectation).

I wish therefore to suggest that the gene for tritanopia, like that for any sex-linked color blindness, occasionally has a severe effect in a heterozygote; and, that when it does, it creates a "tetartanope."

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Discussion

Dr. Edgar Auerbach: You include the response of the rods in your general color perception system. Do you think that that accounts for a certain blue sensation in scotopic vision?

Also, is there some connection with the so-called rhodogenetic theory of Müller?

That is all I have to ask for the time being about this theory, which is very exciting and which has to be digested.

Dr. Gerhard A. Brecher: I think this particular paper is of very great interest. However, I would like to ask Dr. Walls how he can justify putting his schemes on a theory such as the three-color vision theory of Helmholtz-Young, which has been gravely doubted by experimental data from Hering, Tschermak, and Granit.

The one thing I think should be emphasized is that there is a difference between brightness perception and color perception as it was brought forward by Hering about 65 years ago, and as Granit's data also have shown.

However, to base the entire work on an assumption which is doubtful—I am not quite sure if one is justified in going into these intricate schemes which Dr. Walls has presented to us.

Dr. Gordon L. Walls (closing): With regard to Dr. Auerbach's first question: Yes, the reason for showing the rods sharing pathways with the blue cones is for the purpose of helping to account for the fact that in scotopic illumination, feeble illumination below the cone threshold, white light is decidedly bluish, and it is easy to demonstrate experimentally that the hue of this bluishness is exactly that of the elementary blue sensation of photopic color vision.

This is easily accounted for if we only suppose that in weak illumination a few impulses from the rods are able to leak into the color system and establish enough blueness to tinge the white with that blue.

As for his other question, I myself cannot see any relation between my own ideas and the whole body of ideas wrapped up in G. E. Müller's theory.

The last question asked was essentially how I dare to place my scheme on a basis of an outmoded, rejected theory of color vision. I am afraid the questioner misses the point that I am trying to defend the three-component theory, and that everything I have found and seen so far fits that theory and no other.

My branched pathway schema is laid on the three-component theory as a basis because it bears it out. I would challenge him or anyone to interpret these instances of total loss of one color component in these heterozygotes in terms of any other theory.

It is true that the so-called Young-Helmholtz theory, the three-component theory, has lost ground in recent decades, but this is due largely to the inability of people to appreciate the best of the evidence for it, and to see the big holes in some of the other theories.

THE PARTICIPATION OF DIFFERENT TYPES OF CONES IN HUMAN LIGHT AND DARK ADAPTATION*

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Cambridge, Massachusetts

Though it is generally conceded that normal human color vision requires the presence in the retina of at least three different types of cone, it has proved very difficult to demonstrate this directly. Many visual variables show clearly by an inflection or a change in hue the transition from cone to rod function; but the cones, though a highly mixed population, ordinarily display no such discontinuities (Hecht, 1937).

Perhaps the most significant exception to this rule is found in the work of Stiles (1939; 1949a, b). Stiles measured the visibility of a small test stimulus of one color viewed foveally against an adapting field of another color. His measurements display characteristic inflections, like those which ordinarily mark transitions between rod and cone vision, though here involving cones alone. From the analysis of such data, Stiles has derived spectral sensitivity curves for three classes of cone. de Vries (1946) has since reported similar measurements leading to much the same result.

It has long been known that dark adaptation in man and other animals which possess both rods and cones proceeds in two stages. Following a high state of light adaptation, the visual threshold falls rapidly to a first plateau; then the threshold begins abruptly to fall again to a second and final plateau. The first segment is the dark adaptation of the cones, the second that of the rods. The transition from cone to rod thresholds is marked by a more or less sharp break; yet the course of dark adaptation in the cones themselves usually appears to be altogether smooth.

Several workers have adapted the eye to colored lights, thinking that the ensuing dark adaptation might better expose differences among the cones. Wright (1934) and Walters (1942) adapted one eye to monochromatic light, and then followed its increase of sensitivity in the dark by measuring periodically the energy needed in this eye to match the brightness of a constant light of low intensity in the other eye. The measurements were central and involved primarily cone vision. The results displayed a variety of selective effects of adaptation to colored lights, from which the approximate forms of sensation curves could be inferred; but the data obtained in individual experiments displayed no internal evidence of heterogeneity.

Mandelbaum and Mintz (1941) measured the dark adaptation of the human fovea with red, green, and violet test lights, following adaptation with very bright lights of various colors. These measurements also involve primarily the cones alone. The results show small selective effects of colored lights: after exposure to red light, the threshold to red is particularly high, and dark adaptation measured in the red is relatively slow. Exposure to violet light similarly depresses selectively the sensitivity in the violet. Under the circumstances of these experiments, however, all the dark adaptation curves appeared to be homogeneous in form; all of them fall smoothly from the initial reading to the final dark adapted threshold.

We have taken such data to mean that though the retina contains a variety of cones, their spectral sensitivities overlap so widely, and their changes in light and darkness run so closely parallel, that relatively compli-

* From the Biological Laboratories of Harvard University. This investigation was supported in part by funds from the Rockefeller Foundation and the Office of Naval Research.

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cated procedures might be needed to reveal differences in either regard. This is reasonable; for if it were easy to upset the relative contributions of different types of cone to the visual response, any given stimulus would appear to us in many different hues, depending upon the state of the eye and the conditions of stimulation.

The present experiments originated in measurements of dark adaptation with monochromatic test lights in peripheral areas of the retina. When conventional procedures were used, the dark adaptation of the cones appeared to follow its familiar smooth course. When the measurements of visual threshold were spaced very closely together, however, discontinuities appeared. For a time these were thought to be fortuitous variations; but further experiments showed them to be real and reproducible, and to have their source in the participation of several independent mechanisms in cone dark adaptation.

Apparatus and Procedures

All our measurements of the visual threshold were performed with the spectral adaptometer described earlier (Wald, 1945a). In this instrument, 10 wavelengths are isolated with color filters from the radiation of a high pressure mercury arc lamp. The test field subtends an angle of one degree with the eye, and was exposed for flashes of one-fifth second. Its brightness is regulated by means of a pair of annular neutral wedges which rotate in opposite directions so as to compensate each other. A variable fixation point permits the image of the field to fall on any patch of retina within a radius of 12 degrees of the fixation point.

For highly eccentric fixations, the front of this instrument bearing the eyepiece was removed, exposing the test field. The observer's head was held in a chin rest with the eye 80 cm. from the field. Projecting as a radius from the chin-rest as center was a rigid rod, bearing at its end a fixation point, 80 cm. from the eye. The rod could be set by means of a protractor mounted on the chin rest, so as to hold the fixation point at any desired angle with the eye and test field.

For light adaptation we used a 1,000-watt lamp at a distance of 65 cm. from a plano-convex lens (focal length 18 cm., diameter 11.5 cm.). The eye was held at the lens focus in the position of so-called Maxwellian view, in which the surface of the lens appeared to be evenly illuminated. The wavelength composition of the field was regulated with color filters, its brightness with neutral filters.

The brightnesses of both white and colored adapting lights were measured visually with a Macbeth Illuminometer. That is, all such brightnesses were evaluated in terms of the standard white of this instrument. These and other characteristics of the adapting lights are shown in Table 1.

The relative energies of monochromatic light delivered at the test field of the adaptometer were measured with a barrier-layer photocell, calibrated for wavelength sensitivity at our laboratory and by the National Bureau of Standards. All thresholds are reported in this paper in terms of relative energy.

Heterogeneity of Cone Dark Adaptation

Following intense light adaptation, the dark adaptation of an extrafoveal region of the human retina, as already noted, displays clear evidence of rod and cone function. An example of such measurements is shown in Figure 1.

In this instance the eye was highly adapted with green light; the result would not have been very different if white light had been employed. The thresholds measured afterward in the dark with deep red light (691 mμ) fall rapidly from a high initial level to a plateau, attained in about five minutes. There is no further change. The sensation experienced at the threshold is red throughout. This is the dark adaptation of the cones.

TABLE 1

COMPOSITION AND BRIGHTNESS OF ADAPTING LIGHTS

(The source in all cases was a 1,000-watt, concentrated-filament projection lamp, run at about 115 volts. Color filters were of three kinds: Wratten (W), Jena (J) and Corning (C). In all cases a Jena *BG 17* filter was inserted to eliminate heat radiation.)

Filter	Color	Range of Wavelengths (mμ)	Dominant Wavelength (mμ)	Log Brightness (millilamberts)
None	White	All visible	—	5.74
J. *BG 12*	Blue	Below 500	ca. 470	4.20
W. *58*	Green	480–600	540	5.81
C. *3384*	Yellow	Above 490	ca. 580	6.34
C. *3484*	Orange	Above 520	ca. 595	6.15
W. *22*	Orange	Above 550	595	6.14
W. *26*	Orange-red	Above 590	619	5.87
W. *29*	Red	Above 610	632	5.45

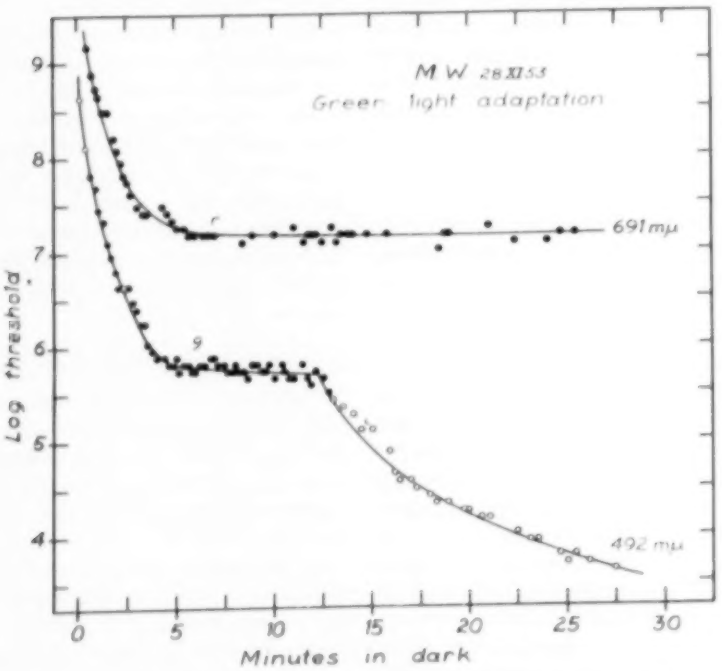

Fig. 1 (Auerbach and Wald). Dark adaptation following five minutes' exposure to brilliant green light (Wratten *58;* log brightness 5.81 millilamberts). Test field: one degree, centered six degrees below the fixation point, and exposed for flashes of one-fifth second. The measurements of threshold were made at 492 mμ in the blue-green, and at 691 mμ in the red. Thresholds are plotted as log relative energy. Solid circles mark thresholds seen as colored; the small letter indicates the color reported (r = red; g = green). Open circles mark thresholds seen as colorless. The entire function at 691 mμ is due to cones. The first segment of dark adaptation measured at 492 mμ also is due to cones, and is seen as colored: the second segment, seen as colorless, is due to rods.

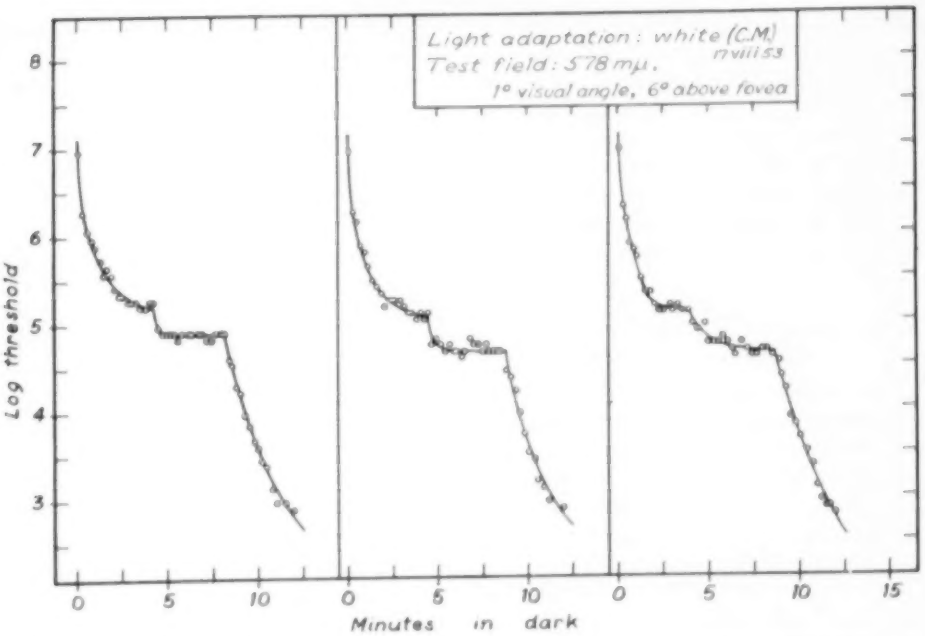

Fig. 2 (Auerbach and Wald). Reproducibility of form of the dark-adaptation curve. Three consecutive experiments on the same day with a single observer. Light adaptation: five minutes in white light, log brightness 5.74 millilamberts. Test field: yellow (578 mμ), one degree, centered six degrees below the fixation point. As usual with a yellow test field, the observer reported all threshold sensations as colorless. All three cone adaptations display distinct inflections in the fourth minute of dark adaptation. In the eighth minute there is a sharp break, marking the transition from cone to rod thresholds.

Rods do not enter such measurements because of their great insensitivity at 691 mμ.

Dark adaptation measured in the same experiment at 492 mμ in the blue-green, a wavelength that readily stimulates both rods and cones, begins with a sharp fall of threshold to a plateau, roughly parallel with that measured in red light. During almost all of this interval the threshold is seen as green. This again is the dark adaptation of the cones. At about 12 minutes, however, there is a sharp break and the threshold begins to fall again; simultaneously there is a loss of color sensations (open circles). This is the dark adaptation of the rods. It ends about 30 minutes later in a second plateau.

In the curves of Figure 1, the cones yield as smooth data as the rods. Under other conditions, however, we occasionally found the cone segment of dark adaptation to display peculiar irregularities of shape, and sometimes more than one plateau.

An example of this type of result is shown in Figure 2. In this case the eye was adapted to bright white light, and thresholds were measured with yellow light (578 mμ). For the first one to two minutes, the cone thresholds fall rapidly. Then they pause or even become constant. Then, at about four minutes, the threshold suddenly falls again, this time to the conventional cone plateau. Here it remains until about the eighth minute, when it breaks again and descends rapidly in the dark adaptation of the rods.

For a time we tended to dismiss such irregularities in the course of cone dark adaptation as fortuitous. As Figure 2 shows, however, they can be highly reproducible. The obvious meaning to attach to them is that they represent an interplay of

responses from several different types of cone, presumably those responsible for color differentiation. If this is so, however, the different types of cone must possess different spectral sensitivities; and it should be possible to alter the shapes of such dark adaptation curves in predictable ways by varying the wavelengths of light adaptation and of the test lights used to measure dark adaptation.

Subjective effects of exposure to intense colored lights

We therefore undertook a study of dark adaptation following light adaptations in selected regions of the spectrum. The principal difference between our measurements and those of earlier workers who had adapted subjects to colored lights involve the intensities employed. Our colored lights are very brilliant, in general of the order of 10^4 to 10^6 millilamberts (see table 1). The selective adaptations which they induced were accompanied by a remarkable series of subjective changes in sensation.*

So for example a subject exposed to our blue adapting light (Jena *BG 12*) sees it for the first few seconds as white. Within about 0.5 minute it turns pink, then within about 1.0 to 1.5 minutes goes over to *bright red;* and so it remains.

Similarly, exposure to an orange-red adapting light (Wratten *26*) yields a momentary sensation of pink, which goes within 10 to 15 seconds over orange and yellow to a *bright green,* maintained as long as the light continues.

Such adaptations to intense colored lights are followed also by strong after-sensations. Immediately following adaptation to the orange-red, a white surface looks deep violet. Following adaptation to the blue, a white surface may look blue for a few seconds, then turns orange, and fades over yellow to eventual colorlessness.

All of this is important in understanding the meaning of the measurements which follow. The sensations experienced during light adaptation and afterward provide immediate and clear evidence that specific classes of color response have been selectively depressed. Adaptation to blue light seems to depress a blue-sensitive mechanism selectively, so that the sensation while the light is on turns to red; but blue light spares relatively the red and green-sensitive systems, so that—following what appears to be a short interval of blue positive after-image—a white surface looks orange (that is, red + somewhat less green). Similarly adaptation to orange-red light selectively depresses a red-sensitive mechanism so that the sensation turns to green. Such orange-red light, as we shall show, almost completely spares the violet mechanism, so that a white surface just afterward looks violet.

Dark adaptation following exposure to intense colored lights

By combining various colors of light adaptation with various colors of test field, we obtained a great variety of cone dark-adaptation curves of different shapes, some simple, some complex. It was possible also to establish the reproducibility of some of the complex functions, as in Figure 2. This provided some ground for confidence in the heterogeneity of the cone function.

Finally we found a combination of conditions which demonstrated this beyond any possibility of doubt. An example is shown at the left in Figure 3. The eye was adapted to orange light of dominant wavelength 595 mμ for five minutes. Then its dark adaptation was measured at 436 and at 621 mμ. These measurements were performed as follows:

In the first few minutes of darkness following light adaptation, thresholds were measured monocularly at 436 mμ alone. A little later these were interspersed with

* It has been known for many years that monochromatic lights change in hue as the brightness is made high (Helmholtz) or low (Bezold-Brücke). We have been unable, however, to find in the literature descriptions of such drastic changes in hue as reported here.

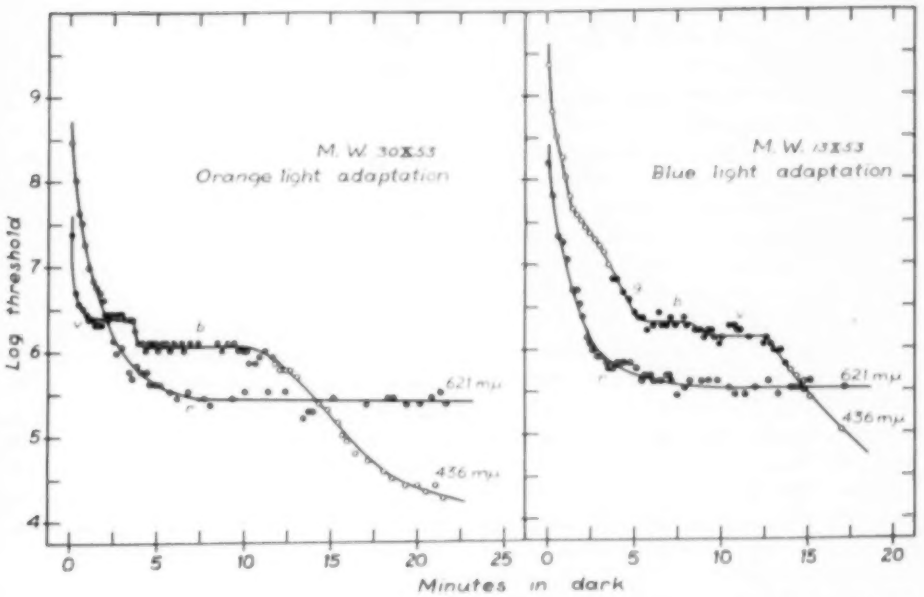

Fig. 3 (Auerbach and Wald). Dark adaptation measured in the violet (436 mμ) and orange (621 mμ), following light adaptation in the orange (Wratten *22*) and blue (Jena *BG 12*). Thresholds seen as colorless are marked with open circles, those seen as colored with filled or partly filled circles, and small letters indicate the color reported: v = violet; b = blue; r = red; g = green. The data at the left provide unequivocal evidence of the responses of at least two types of cone, differing widely in spectral sensitivity. Cone dark adaptation measured at 436 mμ exhibits two plateaus, and two crossings with the measurements at 621 mμ: the first marking the shift of threshold from a violet-sensitive cone to a more general cone response; the second crossing marking the Purkinje shift from cone to rod vision. The measurements on the right exhibit only the second crossing, due to the Purkinje shift; but even here the heterogeneity of cone function is implicit in the peculiar shape of the measurements at 436 mμ, and the sequence of color reports.

measurements at 621 mμ. Then the other eye of the subject was adapted to orange light just as before; and this time measurements were begun at 621 mμ, later interspersing them with readings at 436 mμ. In this way we obtained dark-adaptation curves at both wavelengths simultaneously.

Figure 3 shows that following orange light adaptation, the cones are at first more sensitive to violet light (436 mμ) than to orange (621 mμ). This is just the reverse of the usual relationship found in cone vision. Then at about two minutes of dark adaptation the curves cross, in what may be described as a reverse Purkinje shift; and for the remainder of cone dark adaptation, the threshold is lower to orange than to violet light, the normal cone relationship. At about 14 minutes the curves cross again, this time in the true Purkinje shift, as the thresholds at 436 mμ go over from cones to rods.

In this experiment, the dark adaptation of the cones, measured at 621 mμ, is simple in form; but that measured at 436 mμ is discontinuous. There is a first very rapid fall of threshold to a well-marked plateau, attained in about two minutes; then at about four minutes there is a second sharp fall of threshold to a final cone plateau.

Characteristic color changes accompany this performance. The thresholds at 621 mμ were reported as red throughout the experiment. Those at 436 mμ were reported

first as violet, then as blue, and finally, as the thresholds went over to the rods, as colorless.

Of all these evidences of the heterogeneity of cone dark adaptation, one takes priority over the others. This is the first crossing of the curves, at about two to three minutes in darkness. Before this crossing, the cones were more sensitive to violet than to orange light, a relationship commonly characteristic of rods. Afterward the cones reversed this relationship. That is, for the first two minutes of this experiment the cones exhibited a very different spectral sensitivity from that usually attributed to them, characterized by an unusual sensitivity to violet light. We shall return to this point later.

At the right of Figure 3 a similar experiment is shown, performed with the same subject, but involving adaptation to blue light. Now the thresholds at 436 mμ lie considerably higher than at 621 mμ throughout the course of cone dark adaptation. The curves cross only once, marking the classic Purkinje shift as the thresholds at 436 mμ go over from cones to rods (14 minutes). In earlier times we would have plotted such cone data as a simple pair of parallel lines up to the break in the 436 mμ curve. The measurements at 621 mμ do appear to be simple, and are reported as "red" throughout. The measurements at 436 mμ appear to display minor inflections, but if this were all we should probably have regarded this curve also as simple. The color reports at 436 mμ, however, show that here too we have to deal with a complex succession of responses. At the beginning of dark adaptation, this light, ordinarily seen of course as deep violet, is reported to look *colorless;* later to look *green;* and only toward the end of cone dark adaptation as blue or violet. It is plain that even here, despite the relatively simple geometry of the curve, we are dealing with a complex function.

The violet receptor

The data at the left of Figure 3 yield a first view, in the initial measurements at 436 mμ, of a receptor peculiarly sensitive to violet light. There is no hint of this in the data at the right. Under what conditions does it appear?

This question is answered in Figure 4. Here four dark adaptation experiments have been performed with the same subject as in Figure 3. Each time he was light adapted to a different complex of wavelengths; and his dark adaptation measured at 436 and 621 mμ.

Following adaptation to white light, the cone dark-adaptation curves are relatively simple in shape, and almost parallel. The only reliable evidence of heterogeneity is in the color reports at 436 mμ, which go through a sequence like that just described. At the threshold, the violet test light at first looks colorless, then blue, then green, then blue again, finally becoming colorless as the rods take over. Such sequences vary somewhat from one observer to another, but are remarkably regular in any one subject.

Following adaptation to yellow light containing all wavelengths of the visible spectrum longer than 490 mμ, much the same kind of result is obtained; though in this case the data at 436 mμ seem to describe two cone plateaus.

With adaptation to light longer than about 520 mμ, we obtain the phenomenon introduced in Figure 3. The first minutes of dark adaptation, measured at 436 mμ, involve the function of the special, rapidly adapting, violet-sensitive receptor; and for the first two to three minutes the thresholds at 436 mμ lie below those at 621 mμ.

We can conclude that the special condition which evokes this type of response is adaptation to lights of longer wavelengths than about 520 mμ. Better said, it is protection from lights of shorter wavelength. One can see from Figures 3 and 4 that the dark-adaptation curve measured at 621 mμ changes very little with the quality of light to which the eye is adapted. All our lights adapt very highly the cones sensitive at 621 mμ. The special point is that orange-to-red light almost fails to adapt a violet-sensitive

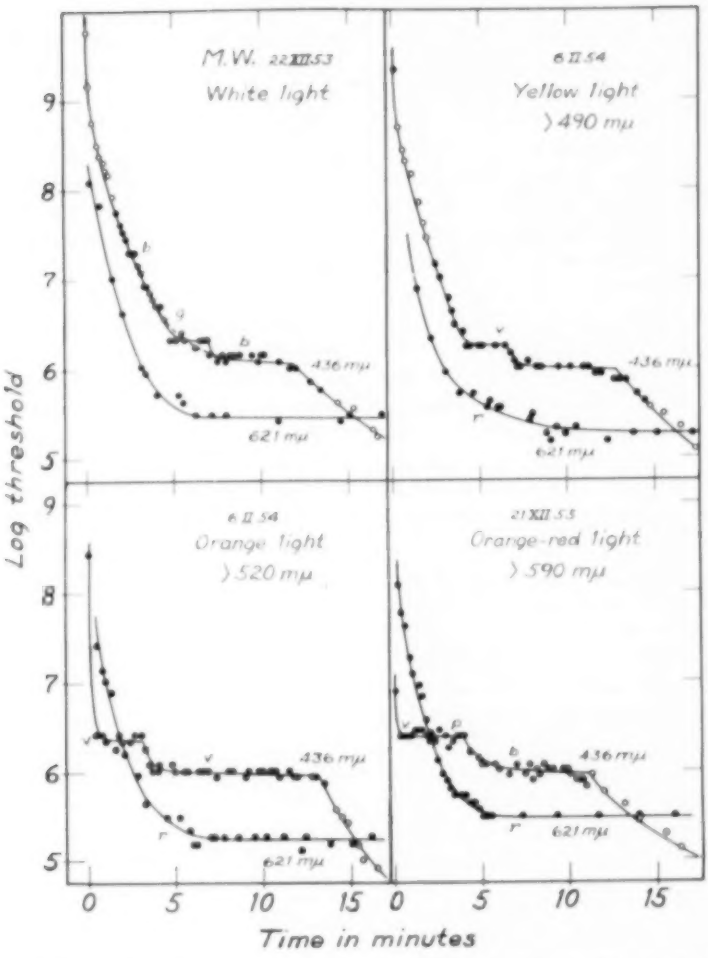

Fig. 4 (Auerbach and Wald). Conditions which evoke the violet receptor. Pairs of dark adaptation curves, measured at 436 and 621 mμ, following adaptation to white and colored lights. Data from a single subject. After adaptation to white or yellow light (Corning *3384*), the dark adaptation curves of the cones are roughly parallel. After exposure to orange light (Corning *3484*), however, the initial portion of dark adaptation measured at 436 mμ is dominated by the violet receptor; and the dark adaptation curves cross twice, as in Figure 3 (left). Adaptation to orange-red light (Wratten *26*) accentuates this effect.

receptor. This is spared to such a degree that its function, measured in the violet, emerges in isolation during the first few minutes of dark adaptation.

Because of this receptor, the spectral sensitivity of cone vision is very different just after adaptation to orange or red light than it is under other circumstances. To measure this difference we have adapted a number of subjects repeatedly to orange or orange-red light, and measured their dark adaptation curves at eight wavelengths available in our spectral adaptometer. A sample of such measurements is shown in Figure 5.

From such families of dark-adaptation curves, we have read off the log thresholds at each wavelength following one minute

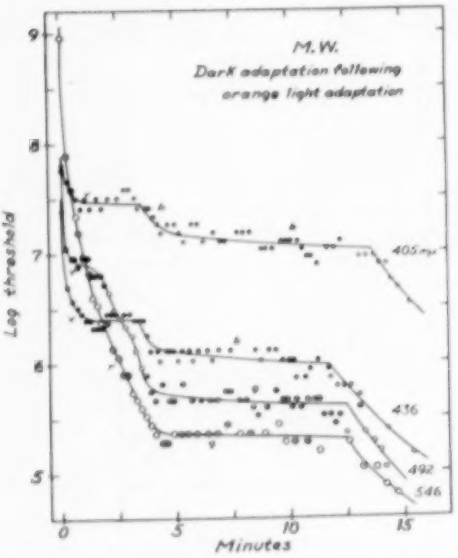

Fig. 5 (Auerbach and Wald). Dark adaptation measured at four wavelengths following light adaptation in the orange or orange-red. The light adaptations were as follows: for the curves measured at 405 and 492 mμ, orange-red light (Wratten *26*). For the curves measured at 436 and 546 mμ: orange light (Wratten *22*). Such families of curves, extended to include other wavelengths, yield values of the threshold at one minute and 10 minutes of dark adaptation from which the spectral sensitivity curves of Figures 6 and 7 were obtained. Dark adaptation measured at 405, 436, and 492 mμ, begins with the violet receptor; and within this series the threshold is minimal at 436 mμ for this element.

and 10 minutes of dark adaptation. To make the data more readily comparable with others in the literature, we present them not as log threshold—the quantity measured—but as log (1/threshold) or (− log threshold), the log sensitivity. In this way we obtain an approximate measure of the spectral sensitivity of cone vision at one and 10 minutes following adaptation to orange-to-red light.

The averaged data of such measurements on seven normal subjects are shown in Figure 6. The curve obtained at 10 minutes of dark adaptation resembles the familiar spectral sensitivity curve of cone vision as measured in the peripheral retina (Wald, 1945b, 1949). It has a broad maximum at about 555 mμ, and an inflection or shoulder in the neighborhood of 450 mμ.

The spectral sensitivity of cone vision at one minute of dark adaptation, however, is altogether different in shape. The principal maximum is in the violet, in the neighborhood of 436 mμ, the sensitivity falling off sharply to either side. This is the spectral sensitivity band of the violet receptor. We see from its shape why lights of wavelength longer than 540 mμ spare this receptor, for at this wavelength its sensitivity has already fallen to a low value. The sensitivity curve

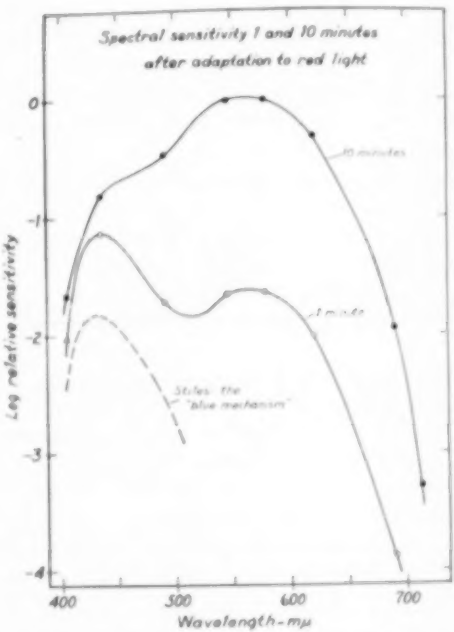

Fig. 6 (Auerbach and Wald). Spectral sensitivity of cone vision measured one and 10 minutes after adaptation to orange-to-red light. Averaged data from seven normal observers. All measurements involve a one-degree field, centered six degrees below the fixation point. The ordinates are plotted as log relative sensitivity (= log 1/threshold, or — log threshold). The maximum of the 10-minute curve has been arbitrarily set at zero (sensitivity = 1). For comparison the curve of Stiles's "blue mechanism" is also included (Stiles, 1949a).

as a whole goes through a minimum at 510 to 520 mμ, and then rises to the broad maximum at about 555 mμ conventionally associated with the cones.

We have, as yet, too few points to define accurately either the shape or position of the spectral sensitivity curve of the violet receptor. It is already clear however that it resembles closely a maximum found by Stiles in the neighborhood of 430 mμ in the spectral sensitivity curve of peripheral cones adapted to intense white light (Stiles, 1949a, fig. 11). From his measurements of the differential threshold of colored test fields against backgrounds of other colors, Stiles has also computed the form of a short-wavelength receptor mechanism which he calls "Blue I" (Stiles, 1949a, fig. 15). This is plotted for comparison in Figure 6. There is no need to labor the point that we seem to be dealing with the same mechanism.*

Indeed the history of this receptor can be traced back to the earliest attempt to derive approximate spectral sensitivity functions for the various types of cone. König and Dieterici (1892), as one outcome of their classic investigation of the color vision of normal and colorblind observers, offered a trio of "Grundempfindungen," or basic sensation curves. The short wavelength member of this set is maximal at about 450 mμ, a little to the red from Stiles's "blue mechanism." Our own data are equally reconcilable with either function.

What sensation goes with this receptor? In the early portions of dark adaptation measured at 405 or 436 mμ following exposure to orange or red light, when one seems to be dealing with this receptor alone, normal observers usually report the threshold sensation as violet, less often as blue (fig. 5). As will appear below, our deuteranopic and protanopic observers have always reported it as "blue." We are therefore faced with some degree of choice regarding this sensation, a choice that has presented a problem since Thomas Young (see the review by Göthlin, 1944).

We prefer to speak of this as the violet receptor because its maximum sensitivity is in the violet; and because when it appears to function alone, most normal observers report the sensation as violet. Another consideration may be relevant: following orange-to-red light adaptation, thresholds measured with violet light usually look violet initially; but later with the descent to the second cone plateau, marking the entrance of other types of cone, they frequently are reported as blue. Examples of this effect appear in Figure 3, in the lower right-hand section of Figure 4, and in the measurements at 405 and 436 mμ in Figure 5. It is as though the violet receptor alone induces a violet sensation, while the mixture with other types of cone changes this to blue.

It might be thought that just as adaptation to red light isolates a violet receptor, adaptation to blue light might isolate a red receptor. Our experiments do not indicate any such result. As shown in Figure 3, adaptation to blue light has little effect on dark adaptation measured at 621 mμ. It does however light adapt very strongly the violet receptor.

We have measured the dark adaptation of several observers at seven to eight wavelengths, following blue light adaptation, in an experiment comparable with that shown in Figure 5. From the family of curves which resulted we have determined the spectral sensitivity of cone vision at one and 10 minutes of dark adaptation. These data are plotted together with those of Figure 6 in Figure 7.

The curve at one-minute dark adaptation after exposure to blue light shows no trace of the violet receptor. Even the shallow in-

* Stiles (1949a) refers to a second "blue mechanism" which, in addition to the peak at about 435 mμ, is relatively sensitive throughout the remainder of the spectrum. This may represent the composite effect of the violet receptor and another type of cone discharging through the same optic fiber.

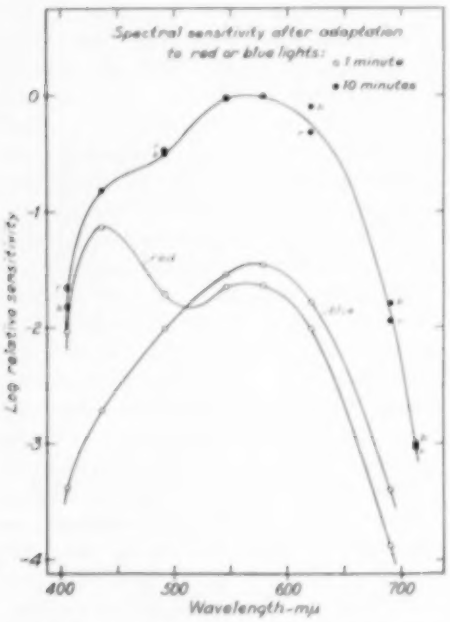

Fig. 7 (Auerbach and Wald). Spectral sensitivity of cone vision measured one and 10 minutes after exposure to intense orange-to-red or blue light. Averaged data from a number of subjects. The sensitivities after orange-to-red light adaptation are as in Figure 6. Those following blue light adaptation were obtained by a similar procedure. The one-minute curves cross at about 510 mμ; below this wavelength the sensitivity is higher following red light adaptation, above this wavelength it is higher following blue light adaptation. Even after 10 minutes in the dark something remains of these differences, as the small letters attached to the solid circles indicate.

flection near 450 mμ in the familiar photopic sensitivity curve is lacking here. There is also another effect: just as red light adaptation spares the violet receptor, blue light adaptation spares—though only slightly—the long wavelength receptors. The two one-minute curves cross at about 510 mμ. Below this wavelength, the sensitivity is higher after red light adaptation; above this wavelength, it is higher after blue light adaptation.

Something remains of these distinctions even after 10 minutes of dark adaptation. Now the sensitivities have drawn close together; 10 minutes in the dark have almost erased the differential effects of red and blue light adaptation. Yet, as the small letters indicate, at short wavelengths the sensitivity tends still to be a little higher after red light adaptation, while at long wavelengths it is a little higher following blue light adaptation.

The 10-minute curve also displays under both conditions the shoulder at about 450 mμ characteristic of the spectral sensitivity of dark-adapted peripheral cones. When first noted in the fovea, this inflection was ascribed to the absorption of light in the blue and violet by the yellow pigment of the macula lutea, xanthophyll (Wald, 1945b, 1949).

The appearance of a similar, though much shallower, inflection in the spectral sensitivity curve of peripheral cones was taken to mean that even well outside the macula, the retina still contains a little of the same pigment. It is apparent from Figures 6 and 7, however, that the inflection in the peripheral curve is caused mainly by the presence of the violet receptor.

Blue light, in adapting this element, removes the inflection completely from the one- minute curve. In this part of the retina, therefore, the inflection is not the negative result of absorption of light by a filter pigment, but the positive result of increased sensitivity introduced by the violet receptor. In the macular region, both factors operate, and to the shallow inflection caused by the violet receptor is added the further inflection due to the macular pigmentation.

The violet receptor in the central fovea

It has been reported on a number of occasions that the central fovea exhibits a condition characterized as "blue-blindness" (König, 1894; Willmer, 1944; Willmer and Wright, 1945). To the unwary this term

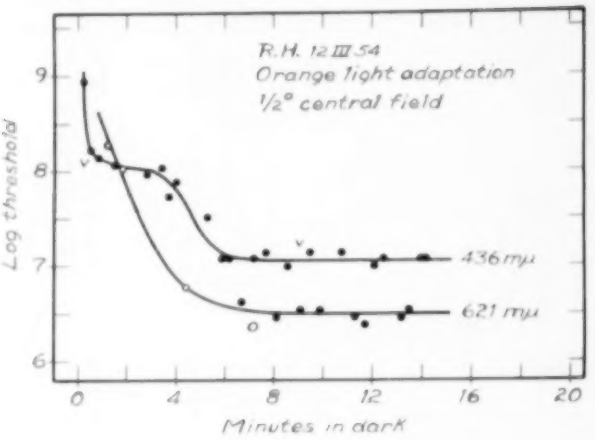

Fig. 8 (Auerbach and Wald). Dark adaptation of a small foveal field following light adaptation in the orange-red (Wratten 26). Measurements at 436 and 621 mμ in a 0.5-degree field, fixated centrally. In this rod-free area of the retina, all the responses are due to cones. The measurements at 436 mμ begin as usual under these conditions with the violet receptor, exhibit the usual double plateau, and cross the curve measured at 621 mμ in what may be called a reverse Purkinje shift.

may imply more than it intends. What it means is that in small areas of the central fovea, all colors can be matched by mixtures of two primary stimuli—460 to 475 mμ and 650 mμ, for example; that is, such small central areas behave in color matching experiments as though dichromatic, and indeed tritanopic. They are however sensitive to blue and violet light, and respond with blue and violet sensations.

We thought it of particular interest to inquire whether the violet receptor appears in a small area of the central fovea. Figure 8 shows that it does. In this instance a 0.5-degree field was employed, fixated centrally. The eye was adapted to intense orange light, and dark adaptation was measured at 436 and 621 mμ. This part of the retina is rod-free, and the thresholds measured are due to cones alone. Just as in the peripheral retina, dark adaptation begins with the thresholds at 436 mμ below those at 621 mμ; and this condition persists until the curves cross at about 1.6 minutes. The dark adaptation measured at 436 mμ also exhibits the two plateaus which characteristically follow orange and red light adaptation.

These are the typical manifestations of the violet receptor. Whatever the cause of "blue-blindness" in the central fovea, it is not the absence of the violet receptor.

Colorblind subjects

To explore further the participation of various types of cone in human vision, we measured dark adaptation under various circumstances in a number of colorblind subjects, chosen and classified by the admirable procedures devised by Farnsworth (1943, 1947; also personal communications). We worked with three protanopes ("red-blinds"), one intensively; and with five deuteranopes. It is well known that protanopes are literally "red-blind" in the sense that their relative sensitivity to red light is far below normal. Deuteranopes, on the other hand, though sometimes referred to as "green-blind," have essentially normal spectral sensitivities.

The experiment of Figure 3 is shown repeated with deuteranopes in Figure 9 and with a protanope in Figure 10.

The result obtained with deuteranopes after adaptation with orange-to-red light (dominant wavelength 619 mμ) is similar to that obtained with normal observers. The dark adaptation measured at 621 mμ displays no new features. At 436 mμ a very rapid fall of threshold is observed, due clearly to the violet receptor; but this appears alone, with no evidence—as in normal observers—of a second plateau or of a change in color sensation during dark

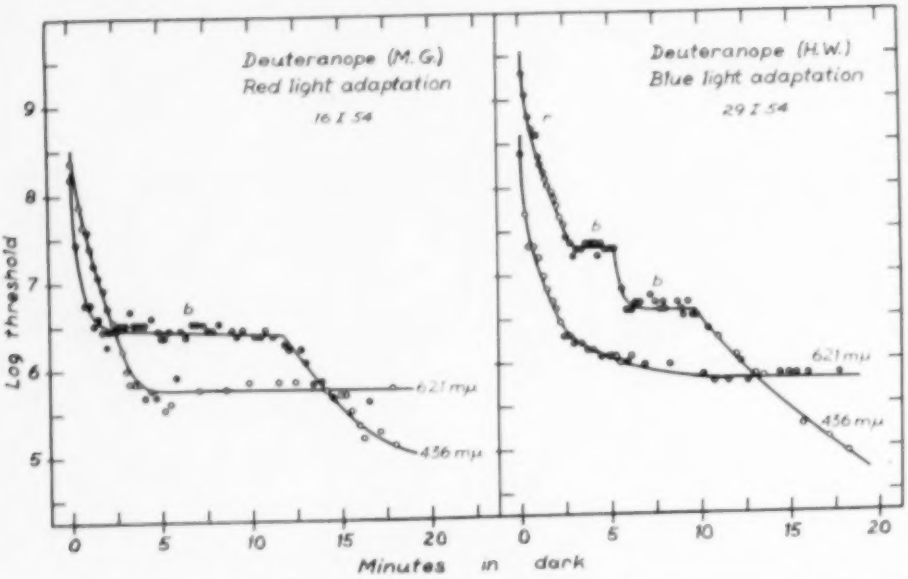

Fig. 9 (Auerbach and Wald). Dark adaptation in deuteranopes following exposure to orange-red (Wratten *26*) or to blue light (Jena *BG 12*). After exposure to orange-red light, the cone adaptation measured at 436 mμ, unlike such measurements in the normal eye (fig. 3), is simple in form, involves no changes in color sensation, and seems to be based upon the violet receptor alone. After adaptation to blue light, the measurements at 436 mμ display two cone plateaus, and a sequence of color reports as though dark adaptation begins with the responses of a red receptor, later mixed with increasing proportions of violet so as to bring the threshold sensations over a neutral point (open circles) to "blue."

adaptation. The color reported is "blue" throughout. The result is as though at 436 mμ one were measuring the dark adaptation of the violet receptor alone.

Following adaptation to blue light (fig. 9, right side), the result with the deuteranopic observer again resembles the normal in its gross features; but the cone measurements at 436 mμ present more striking irregularities than normally, appearing to break cleanly into two segments, each with its plateau. The color reports also are complex. The first thresholds often are reported as "red," though this is of course a violet light; then for a time the thresholds may be said to look colorless; and thereafter blue, until the rods supervene. The course of this function suggests that one may be dealing with the activities of two receptor systems: an initial red, which on later mixing with violet goes through a neutral point ("colorless") to the eventual responses of the violet receptor alone (second plateau), called "blue" by this subject.

Figure 10 shows the results of the same type of experiment performed with a protanope. Following adaptation to orange-to-red light, the dark-adaptation curve measured at 621 mμ is of the usual shape, but is displaced to an abnormal height on the intensity scale, an expression of the subject's red-blindness (Chapanis, 1946-47). The cone dark adaptation measured at 436 mμ is again simple in form, as in the deuteranope, and appears to involve the responses of the violet receptor alone.

Following blue light adaptation (fig. 10, right side), the protanope yields simple dark adaptation curves both at 436 and 621 mμ. The only evidence of heterogeneity in these data is in the color reports at 436 mμ. At the beginning of dark adaptation the threshold

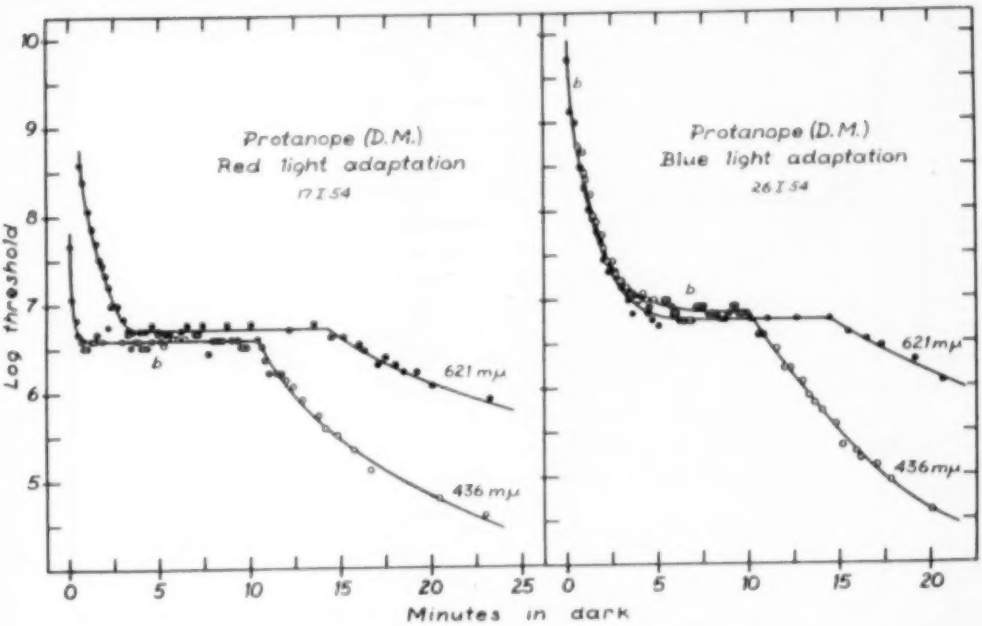

Fig. 10 (Auerbach and Wald). Dark adaptation of a protanope ("red-blind") after exposure to orange-red (Wratten *26*) or blue light (Jena *BG 12*). As usual in such subjects, the cone thresholds to red light (621 mμ) are abnormally high. After orange-red light adaptation, the cone portion of dark adaptation measured at 436 mμ is simple in form, and seems to represent the violet receptor alone. The sensation is reported as "blue." After adaptation to blue light, the measurements at this wavelength are still simple in form, but the color reports go from "blue" to colorless to "blue." The color reports at 621 mμ are very irregular: often colorless, sometimes red, sometimes another color, even blue.

sensation is usually reported as "blue"; later it may be called colorless; and toward the end, as the curve enters the cone plateau, it is again reported as "blue."

Protanopes do not under all circumstances yield such simple dark adaptation curves as shown in Figure 10. With other qualities of light adaptation, and other wavelengths for the measurement of threshold, the dark adaptation curve may display inflections, evidence of the participation of more than one type of cone.

All our observations with protanopes and deuteranopes show that these types of color-blind possess the violet receptor; and that its spectral sensitivity, so far as our measurements go, and its rate of dark adaptation are entirely normal. The abnormalities of such subjects lie—as has been supposed—in other directions. Both classes of color-blind yield evidences, in the form of changes of hue and inflections in the course of dark adaptation—of heterogeneity of cone function. All our observations, though not in themselves adequate to establish this point, are consistent with the view that each of these observers possesses the violet receptor and one other.

Dark adaptation of the far periphery

Toward the beginning of the present investigation, we made a considerable exploration of color thresholds and light and dark adaptation in very eccentric fields. This work was interrupted by the experiments described above; yet it provided another approach to the problem of cone heterogeneity. We shall take this occasion to dis-

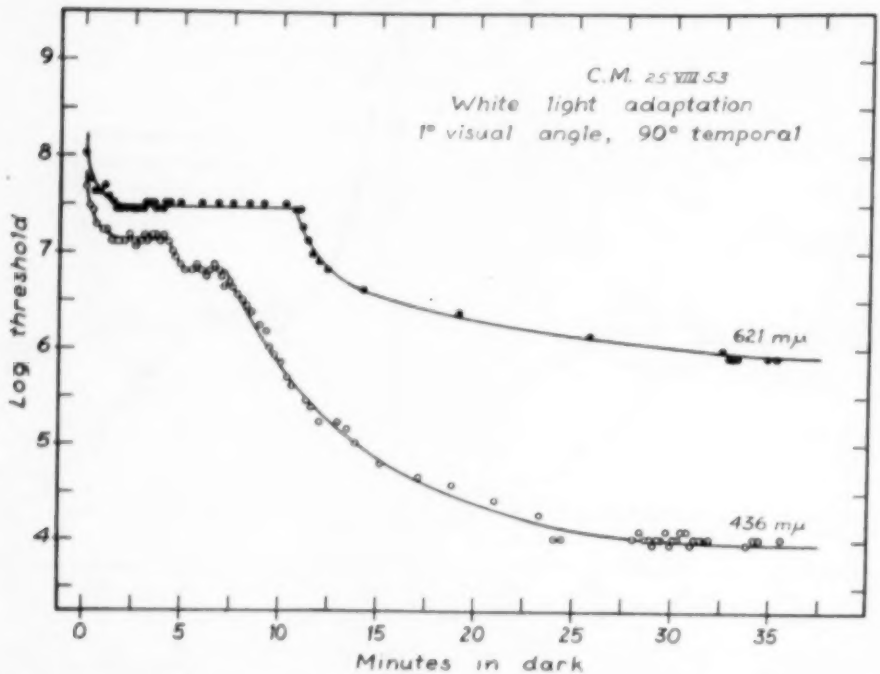

Fig. 11 (Auerbach and Wald). Dark adaptation in the far periphery. This subject was adapted to bright white light, and his dark adaptation measured at 436 and 621 mμ in a one-degree field, centered 90 degrees temporally from the fixation point. At this eccentricity the field looks colorless under all circumstances. The dark-adaptation curves, however, break into typical cone and rod segments; and at 436 mμ the cone segment may have two plateaus. The cone threshold at 621 mμ is abnormally high compared with that at 436 mμ, as in a protanope.

cuss only one example of such an experiment.

Figure 11 shows dark adaptation measurements at 436 and 621 mμ, following exposure to intense white light. The test field, as also the adapting light, were fixated 90 degrees temporally. At this eccentricity—and in general beyond 70 to 80 degrees—lights of all wavelengths appear colorless, even at high brightnesses. In this sense the normal observer is totally colorblind in this part of the retina.

The measurements of Figure 11 show however that both rods and cones are functional. The dark adaptation curves are divided into typical cone and rod segments; and the cone segment measured at 436 mμ seems also to possess two plateaus. The peripheral apparatus for some degree of color discrimination may therefore be present; the lack of color sensations may well be associated with some central failure to exploit the peripheral situation—a central colorblindness.

Apart from the lack of color sensations, measurements with four normal subjects show this region of the retina to be abnormally insensitive to red light. In more central areas—within about 70 degrees of the fovea—following white light adaptation the cone thresholds at 436 mμ lie much higher than at 621 mμ, as shown for example in Figure 4. Here this relation is reversed, as in a protanope, and indeed a protanope

whose red blindness has been accentuated by adaptation to orange-red light, as in Figure 10.

Summary

Following adaptation to very bright white or colored lights, the dark adaptation of the cones displays inflections and changes in color sensation that seem to have their source in the presence in the retina of several different classes of cone. Striking changes in color sensation are experienced also during and immediately after adaptation to very intense red or blue lights.

After exposure of the eye to intense orange-to-red light, the first few minutes of dark adaptation, measured at short wavelengths, are occupied with a receptor maximally sensitive to violet light, and whose excitation induces violet or blue sensations. The maximum sensitivity of this *violet receptor* is near 436 mμ.

Blue light adaptation markedly depresses the response of this unit. Red and blue light adaptations also produce differential effects, though less striking, upon the responses at longer wavelengths.

The violet receptor is present in the central fovea, in spite of its so-called "blue-blindness." It is present also in protanopes and deuteranopes, together perhaps with only one other type of cone.

Measurements in the far peripheral retina —90 degrees from the fovea—show cone function to be present, and reveal a condition resembling protanopia, though no color sensations are experienced.

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Discussion

Dr. Gordon L. Walls: I hope Dr. Auerbach will reconsider a little bit whether he has identified a violet receptor in his curves at all. I should like to point out that red light adaptation would not only spare any receptor sensitive only to short wavelengths, but would also spare the short-wave sensitivity peak of the redness receptor if the redness receptor contains two separate photochemicals, as I believe it does.

Dr. Frederick C. Blodi: I would like to ask two questions about these interesting experiments by Dr. Auerbach and Dr. Wald.

As I understood these charts, the stimulation of cones was done in the fovea or not farther away than eight degrees from the macula.

The first question is whether similar results could be obtained in the far periphery; second, whether in the farthest periphery cone stimulation will give similar results.

We know that there are cones histologically in that area of the retina, but apparently the retina is there color blind.

DR. EDGAR AUERBACH (closing): As to Dr. Walls' question I do not know. This is purely an experimental study, and we did not concern ourselves with any theoretic discussion. I am not at all sure that this red component in the short wavelength range is present but up till now I cannot prove this point.

We think we have demonstrated a receptor which we call the "violet" receptor, because the individual color reports of color-normal subjects were always "violet," and in addition it is in the violet range of the spectrum.

I might add that when we used a mercury-arc lamp as test light, we depended on the spectral wavelengths of the mercury. We could not examine every wavelength we wanted, but only the wavelengths that gave us this monochromatic light in the mercury spectrum.

The peak of the violet receptor may be found a little to the right or left of 436 mμ when more detailed measurements have been completed.

Concerning Dr. Blodi's question, our experiments were made at six or seven degrees from the fovea, to obtain the response of both rods and cones. Some time ago we made a series of experiments in which we examined the whole periphery to 90 degrees from fovea, but we interrupted this work because of our interest in the present experiments.

Beyond 70 degrees from the fovea we always observed a higher threshold to the red than to the blue. This is from approximately 70 to 90 degrees. From the fovea to 70 degrees the blue has a higher threshold. This was very interesting and was very regular. This phenomenon we could always reproduce.

Now, in doing this present experiment with the protanopes, we remembered this old experiment of ours, because this appearance of a higher threshold to the red in the periphery of color-normal persons is approximately the same as in protanopes.

The next question asked by Dr. Blodi concerned the color reports. It is at this part of the periphery that there is no color report. This means that the individual, from this point of view, is subjectively completely color-blind; but as you see in these curves, there is a cone and a rod portion. This means there are cones still active, and even the double plateau 436 mμ was still present. This means that even different cone receptors were active.

SETUP FOR THE PHOTOGRAPHY OF THE ANTERIOR SEGMENT OF THE RABBIT EYE*

WILLIAM STONE, JR., M.D., AND LEONARD REYNOLDS
Boston, Massachusetts

This paper presents a setup which is utilized in this laboratory at the Massachusetts Eye and Ear Infirmary for the rapid photography of the anterior segment of rabbit eyes. Its value is that it is unobtrusive, permanently set up, and can be moved into working position quickly. The rabbit's head is held immobile and can be rotated to any angle.

Its component parts are readily available or easily constructed; and it can be placed at the corner of most laboratory benches.

It is possible for one person to place an animal in position and take a picture of its eye within two to three minutes. Previously, the same procedure in this laboratory involved several persons maintaining the animal in the correct position and taking the picture. The procedure usually took 20 to 30 minutes. We now take postoperative serial photographs with nearly the same facility as making notes. Human photography can be done in the same area. The adjustable box is also suitable for slitlamp microscopy.

DESCRIPTION OF COMPONENT PARTS

Figure 1 shows the setup in working position. The handle (A) makes it possible to rotate the box around a horizontal axis through 180 degrees and around a vertical axis through 360 degrees. A twist of the

* From the Massachusetts Eye and Ear Infirmary. This work was supported by the Office of Naval Research (Contract Nonr 1173-01) and the Boston Host Lions Club.

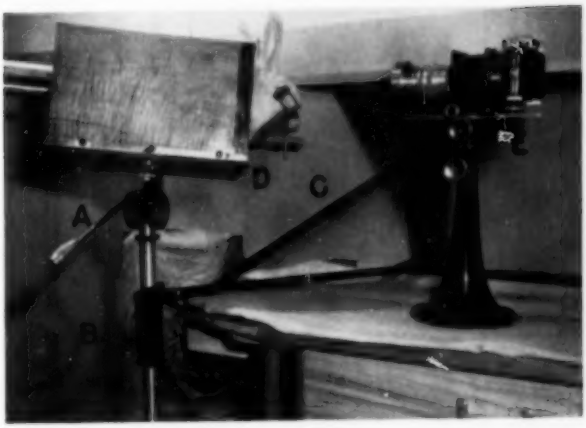

Fig. 1 (Stone and Reynolds). Setup for photography of rabbit eye in working position. (A) Handle and swivel-head for raising and lowering box. (B) Guide support for column and pinion. (C) Swinging arm and brace. (D) Rabbit box on platform. (E) Sliding camera mount.

(Figure 2 is shown on the next page)

handle locks the box in the desired position. This column with rack and swivel head is commercially available (A*). A pinion and support guide (B) for the column raises and lowers the box.

The pinion and support guide is specially constructed to lend stability to the setup. Figure 2 is a diagram of this construction.

* (A) Quick Set Inc., 5121 North Central Park Avenue, Skokie, Illinois.

Figure 1-C is a swinging arm. Heavy braced steel is utilized to minimize the movements occasioned by the rabbit's respirations (fig. 2 and fig. 3). Figure 1-D and Figure 3 show the platform supporting the rabbit box.

The rabbit box is of the usual laboratory variety with a slightly modified head rest (fig. 4). An L-shaped bar is placed through the rabbit's mouth, and supported in a slot

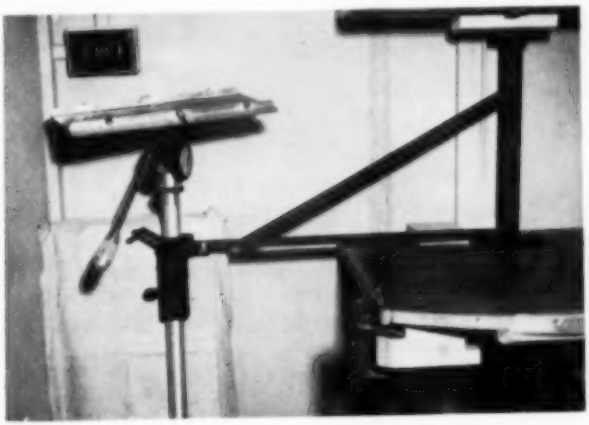

Fig. 3 (Stone and Reynolds). Rabbit photographic setup without rabbit box. Box platform is rotated against wall.

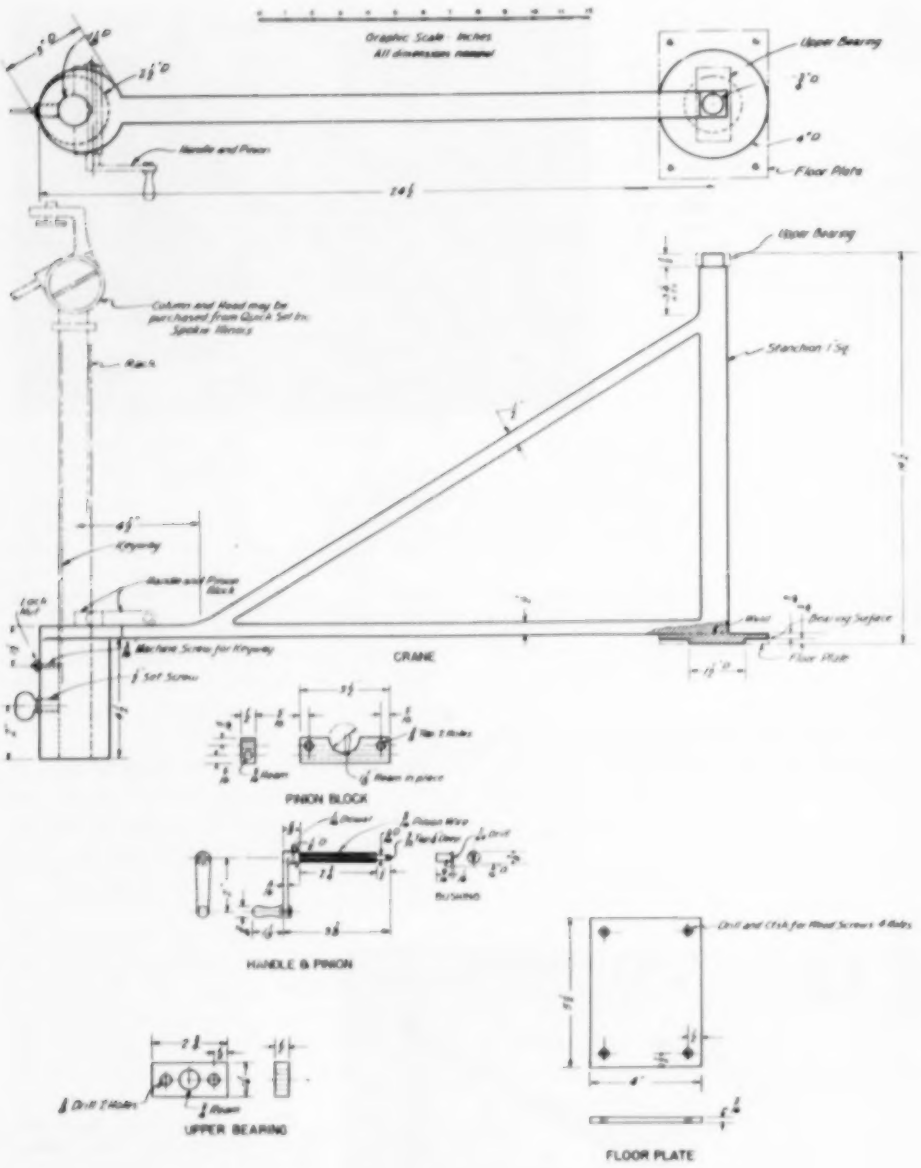

Fig. 2 (Stone and Reynolds). Diagram of construction of support-guide with pinion.

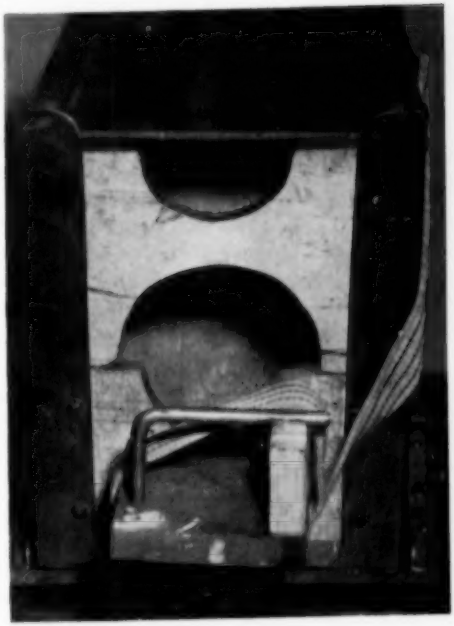

Fig. 4 (Stone and Reynolds). Animal box, showing modified headrest. L-shaped brace passes through animal's mouth, and fits into slot at either end. Webbing wraps around rabbit's head and metal clasp fits into slot on upper edge of box.

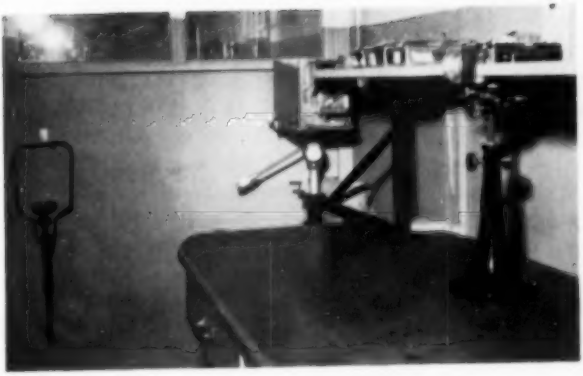

Fig. 5 (Stone and Reynolds). Rabbit photographic setup when not in use. Box rotated against wall. Camera moved into slot in overhanging shelf. Human headrest hung on wall.

at either end. The rabbit's head is then locked in position by means of a piece of webbing and secured with a metal clasp in a slot.

The camera is mounted on a commercially available sliding slitlamp mount (E*) (fig. 1-E). Figure 5 shows the setup when it is not in use. The rabbit box and support are rotated against the wall and the camera is moved into a slot in the overhanging shelf. The human headrest can be seen hanging on the wall and is placed in a fitting at the edge of the bench when in use.

213 Charles Street (14).

* (E) Bausch and Lomb Company.

EFFECT OF ALCOHOL ON BINOCULAR VISION*

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It is well known that alcohol produces double vision. This has been attributed by Colson[1] and Powell[2] to two factors: (1) Changes in phoria and (2) loss of the power to converge. However, it is generally believed that there are at least four separate mechanisms concerned with the angular position of the eyes, any one of which, if disturbed, could contribute to diplopia. These are: (1) Phoria, (2) voluntary convergence, (3) reflex (accommodative) convergence, and (4) the fusion reflex. All four mechanisms are closely integrated to achieve single vision. The fusion reflex in particular tends to adjust the position of both eyes in such manner as to project the images on the corresponding "Panum areas." This is subjectively perceived as single vision.

Previous experiments on the influence of alcohol on binocular vision were made with the prism vergence tests.[1-3] In such tests, one cannot separate the convergence mechanism from the fusion reflex.[4] Therefore, it is not known whether the binocular fusion reflex is impaired by alcohol independently of phoria or convergence changes.

The purpose of the present investigation was to study the effect of alcohol on the power of binocular fusion and to attempt a separation of the four components which contribute to diplopia. In addition, it was intended to solve the disagreement existing in the literature as to the direction of the change in phoria.

Colson[1] and Powell[2] measuring phoria at an object distance of six meters, found a progressive development of esophoria with increasing amounts of alcohol. Powell, also using an object distance of 33 cm., found progressive exophoria. No satisfactory explanation of these different trends of phoria development was offered.

Charnwood[3] reported little or no change in vertical phoria. Furthermore, previous workers[1-3] have merely related in a rather qualitative manner the subject's visual behavior to the amount of ingested alcohol. In this study, blood alcohol and urine alcohol concentrations were quantitatively determined.

METHOD

The subjects for these experiments were 14 male medical students between 21 and 28 years of age. The experiments began at 7:00 P.M. after a light evening meal. Visual acuity, convergence, phoria, and fusion power were determined in the manner to be described. The subjects were then given 40 ml. of an 87-proof blended whiskey, mixed with 99 ml. of either water or ginger ale, and this was repeated every half an hour.

At hourly intervals after the start of the first drink, the subject repeated the vision tests. At the end of each of these tests, two ml. of blood was removed from the antecubital vein and a urine sample was obtained. In this manner, blood and urine samples were taken at hourly intervals, whereas the drinks were given at one-half hour intervals. This routine was continued until the subject vomited or was incapable of co-operating in the conduct of the experiment.

Visual acuity was determined with a Snellen chart at a distance of six meters. Convergence was determined by having the subject bring an upright metal rod progressively closer to his eyes until diplopia

* From the Department of Physiology, Western Reserve University School of Medicine. Part of this work was done in fulfillment of the research project teaching program of the curriculum at Western Reserve University School of Medicine.

resulted. This distance was then measured along with the interpupillary distance and the angle of convergence was calculated. Heterophoria was determined at a distance of six meters with a Maddox rod.

The method used for the determination of fusion power has already been described.[5] The following modifications were introduced for the alcohol experiments.

In a well-illuminated room (50 foot-candles) the observer looked at a black screen in the center of which was a small light source. Three such screens were used, at a distance of six meters, 82 cm., and 33 cm. White lines divided the screens into squares such that the visual angle between lines was the same for all screens (approximately 0.5 degrees).

The right eye was occluded with a photographic shutter (Wollensack Alphax No. 2). With an exposure time of 20 milliseconds, the shutter was released by an electronic timing device at a frequency of three per second. In this manner, fusion was tested by the interrupted presentation of the normal binocular fusion stimulus to the right eye in a series of brief flashes, while the same stimulus acted on the left eye without interruption. The stimulus-evoking fusion was thus provided by the indentical contours of the practically unimpaired normal binocular visual field.

The subjects were first placed in front of the six-meter screen and the right eye occluded for 20 seconds with the shutter. Upon one single shutter release of one-fiftieth second duration, the subject was asked to report the position of the double image of the light source on the screen. This was used as a measure of vertical and horizontal phorias, in addition to the Maddox measurements.

The subject was then tested in the same manner with a frequency of three exposures per second. The time necessary for the attainment of complete fusion was recorded by the subject with an electric stopclock. Five such readings were taken and averaged. A significant change in this fusion time in subsequent tests, with the same frequency of stimulus presentation, was taken as a measure of the change in fusion power.

This same procedure, including the phoria determination, was then repeated in front of the 82-cm. screen and the 33-cm. screen. The six-meter and 33-cm. distances had been used by previous workers;[1, 2] the 82-cm. distance was added in this experiment in an attempt to locate a distance at which there was little or no change in phoria.

The oxalated blood samples and the urine samples were refrigerated, and subsequently analyzed for alcohol concentration by the method of Sunshine and Nenad.[6]

Results

The experimental data of 14 subjects are graphically presented in seven figures.* The first three figures show the effect of alcohol on binocular fusion time and the change in phoria. Since fusion time is known to be related to the degree of heterophoria,[5] the subjects were grouped according to the initial type of phoria they displayed without alcohol ingestion at six meters observation distance. Reciprocal fusion time is plotted as ordinate against blood alcohol content as abscissa.

The values which were to be represented on the ordinate were grouped according to the blood alcohol level at the time of their measurement. These arbitrary alcohol groups include those values in 0, 0.01-0.05 percent, 0.06-0.10 percent, 0.11-0.15 percent, and 0.16-0.20 percent ranges. The ordinate values in each of these groups were averaged as were the corresponding alcohol concentrations. Fusion times was plotted as reciprocals in order to represent fusional failure as zero and not infinity.

Figure 1 illustrates the influence of alco-

* The tables of individual subject data were omitted for space conservation. They are available on request from the Department of Physiology, c/o G. A. Brecher, M.D.

hol on the fusion time of five subjects, who were initially exophoric at six meters observation distance. The diagram on the right side of the figure shows the position of the visual lines at three observation distances before alcohol ingestion. The arrows indicate at each observation distance the direction of the phoria changes with increasing intoxication.

It is noted that alcohol diminished the exophoria at six meters until the subject became orthophoric and finally even esophoric. At 82 cm. exophoria decreased until the subjects became nearly orthophoric whereas at 33 cm. the degree of esophoria became larger.

On the left side of Figure 1, the reciprocal of fusion time is plotted against the blood alcohol content. With this type of plotting, an upward trend of values signifies a shortening of fusion time and a downward trend a lengthening. A zero value means that binocular fusion could not be accomplished.

It is noted that at six meters distance, after a consumption of a small amount of alcohol, fusion power did not change significantly. This is apparently due to the fact that the exophoria lessened and the eye approached an orthophoric position. Thereafter, fusion time became progressively longer with increasing alcohol dosages as demonstrated by the steep decline of the solid curve.

At 82 cm. observation distance, the fusion time became consistently longer with higher blood alcohol levels (broken line). With near vision (33 cm., dotted curve), fusion time became longer and all subjects failed to fuse entirely at blood alcohol contents above 0.1 percent.

The behavior of the initially orthophoric group of observers is illustrated in Figure 2. The type of plotting and the symbols used are the same as in Figure 1.

At six meters distance, the visual axes converged increasingly with rising alcohol levels. At 82 cm., there was a very slight development of esophoria; whereas, at 33 cm. distance, exophoria developed.

In the left part of Figure 2, it is seen that fusional power decreased at all distances. Since the orthophoric group is represented by only three subjects, the individual variations have a large effect on the smoothness of the curve. This is especially true in the case of the last value on the 33-cm. curve, since only one subject in this group reached an alcohol level above 0.15 percent. It can be assumed that this part of the curve would

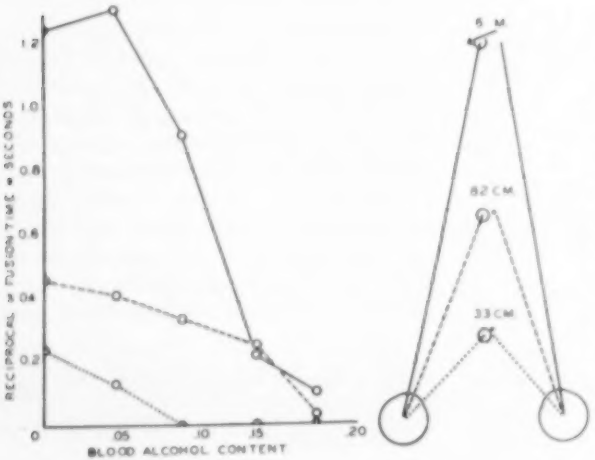

Fig. 1 (Brecher, Hartman, and Leonard). *Exophoric group, effect of alcohol on fusion time and phoria.*

(Left) Changes in the reciprocal of the binocular fusion time in seconds with increasing blood alcohol content (percent). Solid line: six meters viewing distance; broken line, 82 cm., dotted line, 33 cm.

(Right) Changes in horizontal phoria from zero to maximum blood alcohol content (horizontal scale exaggerated ×10).

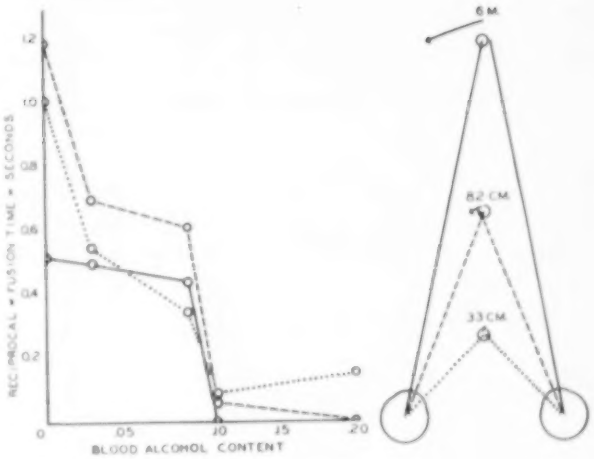

Fig. 2 (Brecher, Hartman, and Leonard). *Orthophoric group, effect of alcohol on fusion time and phoria.*

(Left) Changes in the reciprocal of the binocular fusion time in seconds with increasing blood alcohol content (percent). Solid line: six meters viewing distance; broken line, 82 cm., dotted line, 33 cm.

(Right) Changes in horizontal phoria from zero to maximum blood alcohol content (horizontal scale exaggerated ×10).

have been lowered if the other subjects had been able to reach this level.

Figure 3 represents the data of the initially esophoric group (six subjects). The type of plotting corresponds to that used in Figure 1.

At six meters, the normally converged axes of these subjects converged still more with alcohol. At 82 cm. the esophoria increased by a very small amount; whereas, at 33 cm. the esophoria decreased, went through a stage of orthophoria, and turned into an exophoria with greater alcohol intoxication. The fusion time became progressively longer at all distances, with alcohol ingestion.

In all three groups of subjects, it was found that binocular fusion could still be retained at the 82-cm. observation distance, while it was already lost at distant (six meters) and near (33 cm.) vision.

Figure 4 depicts the average reciprocal fusion times of all 14 subjects plotted against the blood alcohol content. It is noted that the fusion times became progressively longer with increasing alcohol intoxication.

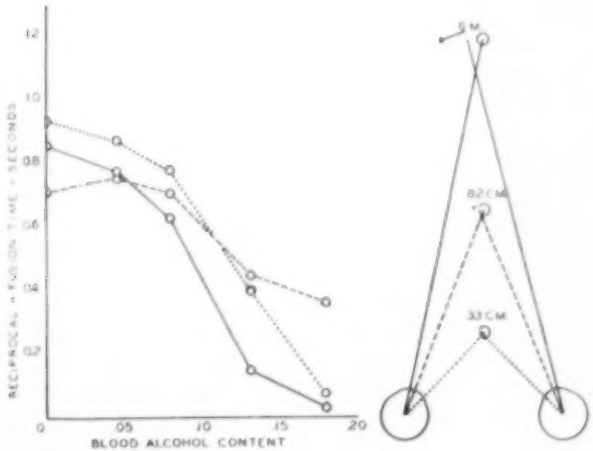

Fig. 3 (Brecher, Hartman, and Leonard). *Esophoric group, effect of alcohol on fusion time and phoria.*

(Left) Changes in the reciprocal of the binocular fusion time in seconds with increasing blood alcohol content (percent). Solid line: six meters viewing distance; broken line, 82 cm., dotted line, 33 cm.

(Right) Changes in horizontal phoria from zero to maximum blood alcohol content (horizontal scale exaggerated ×10).

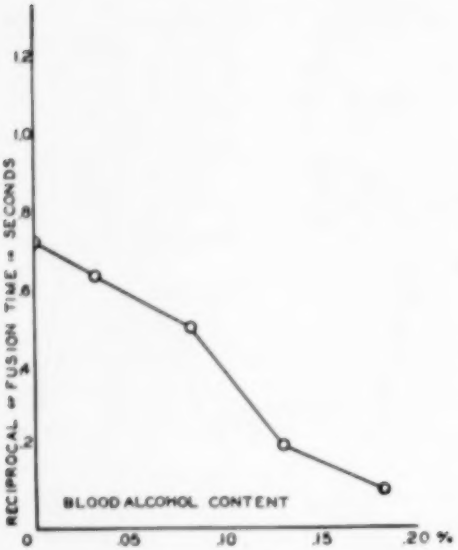

Fig. 4 (Brecher, Hartman, and Leonard). *Effect of alcohol on binocular fusion.*
Average of results for three groups and three distances. Changes in the reciprocal of the binocular fusion time in seconds with increasing blood alcohol content (percent).

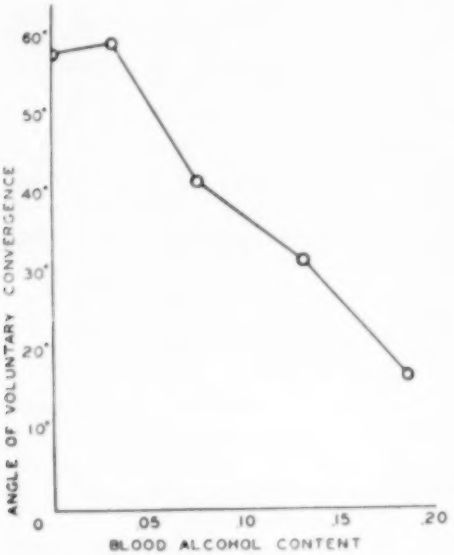

Fig. 5 (Brecher, Hartman, and Leonard). Changes in the angle of voluntary convergence (degrees) with increasing blood alcohol content (percent).

Figure 5 illustrates the effect of alcohol on voluntary convergence. The angle of voluntary convergence in degrees is plotted on the ordinate and the blood alcohol content on the abscissa. The data represent the average of all 14 subjects. It is noted that with mild alcohol ingestion up to 0.03 percent, there was no impairment of the power to converge voluntarily, whereas this power was progressivly impaired at higher alcohol levels.

Figure 6 shows the effect of alcohol on vertical phoria at different observation distances. On the ordinate, the degree of change in hyperphoria is plotted in prism diopters (hyperphoria for the right eye above the horizontal line, for the left eye below the horizontal line).

According to the three observation distances, there are three separate graphs in Figure 6, for the screen distance of six meters (top), 82 cm. (middle), and 33 cm. (bottom). Blood alcohol content is plotted on the abscissa. The closeness of the points to the horizontal of zero prism diopters for all three screen distances illustrates that vertical phoria was not significantly changed by alcohol intoxication. This confirms the observation of Charnwood.[3]

Discussion

The results of these experiments contribute to the solution of the question, whether the binocular fusion reflex as such is impaired with alcohol intoxication, thus causing alcohol diplopia. The strength of this reflex (binocular fusion power) was determined by measuring the time necessary to elicit it when normal binocular fusion stimuli were presented in the form of brief flashes, at a frequency of three per second. The time measured, called "fusion time," was taken as index of the binocular fusion power.[5]

It was found that regardless of directional

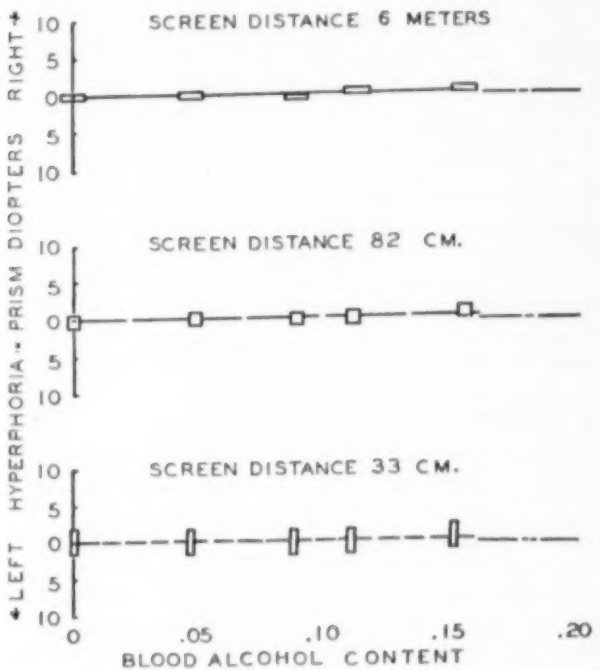

Fig. 6 (Brecher, Hartman, and Leonard). Changes in vertical phoria with increasing blood alcohol content (percent). Solid line, trend line at six meters viewing distance; broken line, 82 cm.; dotted line, 33 cm. Dot-and-dash line, zero hyperphoria.

and quantitative changes in phoria, the average fusion time became progressively longer with increasing blood alcohol levels. From these findings, it must be concluded that alcohol impairs directly the functioning of the binocular fusion reflex.

The power of voluntary convergence was also impaired with alcohol intoxication. However, this cannot be the cause of the failure to fuse, since the reflex of binocular fusion is tested under experimental conditions in which voluntary efforts were found to have very little effect.

The experiments elucidate, futhermore, the controversial findings of Colson[1] and Powell[2] as to the heterophoria changes with alcohol. This was accomplished by measuring phorias at far, near, and intermediate distances and by following the changes to higher degrees of intoxication than previously studied.[1,2] It was found that regardless of the initial type of phoria (eso or exo) the visual axes always converged with alcohol at six meters observation distance, diverged at 33 cm. but changed only slightly at 82 cm. With high alcohol concentrations, all subjects approached orthophoria or a small degree of esophoria at 82 cm.

Unfortunately, the intermediate distance of 82 cm. had to be chosen in these experiments without previous knowledge of the optimal distance, at which phoria remains unaltered. The results show that a screen distance slightly less than 82 cm. would have been better in order to find the point at which there was no phoria change at all with alcohol. An attempt to determine graphically this particular distance is shown in Figure 7.

The change in horizontal phorias was expressed as the ratio: diopter/percent blood alcohol content, for each of the three observation distances. The values for the group which was initially exophoric at six meters is symbolized by an "X," the orthophoric group by a point, the esophoric group by an upright cross, and the average of all groups

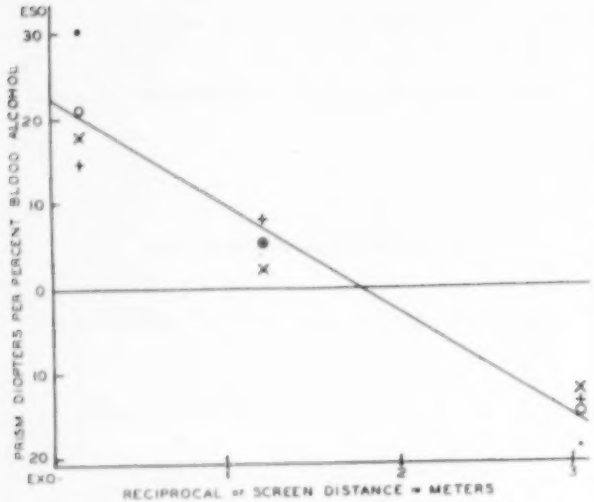

Fig. 7 (Brecher, Hartman, and Leonard). Ratio of change in horizontal phoria with increasing blood alcohol content (prism diopters/percent of blood alcohol) as a function of the reciprocal of the viewing distance in meters. (X) exophoric group; (.) orthophoric group; (+) esophoric group; (o) mean of three groups. Intersection of trend line with horizontal (axis of abscissae) indicates reciprocal of viewing distance (56-60 cm.) at which horizontal phoria would not change with increasing blood alcohol content.

by an open circle. The change of heterophoria is plotted as ordinate in prism diopters per percent of blood alcohol content against the reciprocal of the screen distance, in meters, as abscissa.

The upper left values illustrate that at six meters screen distance, marked esophoria developed, at 82 cm. a very small amount of esophoria and at 33 cm. distance marked exophoria. Connecting the average values with a straight line one notes that this line intersects the zero line of "no phoria change" at about 56 to 60 cm.

From these findings, it may be concluded that the resting position of the eyes at which alcohol does not cause phoria to change is at a distance of about 60 cm. The fact that the eyes assume this "position of rest" could be explained by the decrease or removal of neuromuscular co-ordination through the action of alcohol.

Placing the "position of rest" of the eyes at a viewing distance of about 60 cm. implies that the relative divergence and diminished accommodation for fixation at greater distances are active processes. Duke-Elder[10] has summarized the evidence and arguments for a divergence center, and recognizes negative as well as positive relative accommodation.

In the near reflex, according to Adler,[11] "the contraction of the pupil begins only at the moment that the fixed object is at a distance of 0.5M., and not at the moment that accommodation begins."

The finding of a "position of rest" of the eye axes under alcohol influence agrees with the conjecture of Luckiesh and Moss[7] "that the state of repose would be coincident with some compromise position between the extremes of distant and near vision."

That the accommodative mechanism[12] may be linked with this "position of rest" of both eyes could also be deduced from the "lead of accommodation" of 0.75D. at six meters[7] and the well-known phenomenon of "sky myopia" in the order of one to one and a half diopters.

For a mean interpupillary distance of 6.62 cm., convergence at 56 cm. corresponds to a mean angle of the visual axis with the sagittal plane of 3.4 degrees. Unfortunately, no measurements of the angle (alpha, gamma, kappa, lambda) between the optical

and visual axes were made for these subjects: the usual value for emmetropic subjects is about five degrees. Thus, in the "position of rest," the optic axes are more nearly parallel than the visual axes.

As reported by both Colson[1] and Powell[2] there is a decrease in convergence ability with increasing concentrations of alcohol. The decreases in the convergence angles found here are considerably greater than those reported by Colson.[1] This is probably due to the fact that greater quantities of alcohol were consumed by the subjects in these experiments.

Figure 5 shows that at higher levels there is an almost linear relationship between blood alcohol concentration and the angle of convergence. If this line could be extrapolated the angle of convergence would then be zero at a blood level of about 0.26 percent. From this it might be assumed that at this alcohol level there would be complete failure of voluntary convergence. A similar extrapolation on Figure 4 indicates that fusion power would be lost at the same blood alcohol concentration. This concentration is roughly that at which all voluntary muscular control is lost.[8, 9]

It is interesting to note that legal intoxication is generally accepted as a 0.15-per cent alcohol concentration. In the 0.05-0.15 percent range an individual is considered "under the influence."[8, 9] From these experiments it can be seen that at the level of definite intoxication, fusion power and convergence are very markedly impaired. At the 0.05-percent level neither of these mechanisms seem seriously handicapped but, somewhere between 0.05 and 0.15 percent, all subjects showed definite impairment. Most of the subjects showed no significant change in visual acuity over the entire range of blood alcohol concentration. In three cases there was a measurable decrease in visual acuity at the higher alcohol levels.

Summary

The question was studied whether alcohol diplopia can be attributed to change in phoria and loss of convergence power as previously postulated or to an impairment of the binocular fusion reflex. This was tested in 14 subjects with a new method in which the strength of binocular fusion could be determined by measuring the time necessary to accomplish binocular fusion.

It was found that diplopia is caused by the progressive impairment of the binocular fusion reflex with rising levels of blood alcohol content. This impairment of the fusion reflex occurs regardless of changes in phoria and of the weakening of voluntary convergence. The decrease in binocular fusion power is due to an impairment of the general neuromuscular co-ordination by alcohol which at the same time affects the position of the visual axes and diminishes the power of voluntary convergence.

Phoria and fusion power were measured at 600-cm., 82-cm., and 33-cm. observation distances. With rising alcohol levels, marked esophoria developed progressively at 600 cm., only little esophoria at 82 cm., but marked exophoria at 33 cm. This indicates that the visual axes come to a "position of rest" at a distance slightly less than 82 cm. (about 60 cm.) under strong alcohol intoxication.

This evidence supports the hypothesis that binocular vergence is normally controlled by two different mechanisms, a convergence and a separate divergence mechanism, since the removal of these mechanisms by alcohol cause the eyes to assume an intermediate or neutral position.

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The authors want to thank Dr. Irving Sunshine, Department of Pathology, School of Medicine, Western Reserve University, for his help and co-operation in the blood and urine alcohol determinations.

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AN EXPERIMENTAL STUDY OF ELECTRORETINOGRAPHY*

I. The electroretinogram in experimental animals under the influence of methanol and its oxidation products

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Several investigators have attempted to elucidate the site of generation of the electroretinogram by studying the effects of drugs on the electroretinogram and optic-nerve discharge. Extensive studies of this sort have been carried out on amphibia and reptiles by Granit[1] and Therman[2] and on mammals by Noel.[3] We, on the other hand, have been primarily interested in assaying the effect of various toxic substances on the visual system as manifested by changes in the electroretinogram and optic-nerve discharge. As a direct result of this investigation, information was also obtained which is of value in considering the problem of the site of origin of the various electroretinogram potentials.

This latter approach is of particular value when the retinal toxicity of a substance is due to its conversion into a toxic product by metabolic processes. In such a case, the suspected intermediates can be tried and the most likely agent can be picked out on the basis of the relative concentrations necessary for an effect.

The problem of human methanol poisoning falls into this category. In previous work reported from this laboratory[4] efforts were made to identify the proximal toxic agent in methanol poisoning. The chief candidates were methanol and its oxidation products, formaldehyde and formate. Experiments in vitro on the metabolic activity of the surviving retina showed that for every enzyme activity tested, the inhibitory potency was formaldehyde > formate > methanol. These experiments showed that anerobic glycolysis was by far the most sensitive enzyme system and later work[5] localized the inhibition in the hexokinase portion of glycolysis.

Noel[3, 6] has shown that other poisons of anerobic glycolysis, such as azide and iodoacetic acid, also cause retinal effects which, in

* From the Laboratory in Research in Ophthalmology and Department of Physiology Western Reserve University, and the Ophthalmologic Service, Department of Surgery, University Hospitals of Cleveland. Supported by a grant from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service.

his experiments, are signaled by marked changes in the pattern of the electroretinogram and optic-nerve discharge. These effects occur in concentrations considerably below the whole animal toxic dose. The action of iodoacetic acid has been attributed to specific inhibition of retinal anerobic glycolysis. It has been further claimed that anoxia affects other components of the electroretinogram than iodoacetic acid. On this basis the electroretinogram components of the cat and rabbit are classified into two groups, those resistant to anoxia and those resistant to iodoacetic acid. The cat b-wave* and the rabbit a-wave are most resistant to iodoacetic acid and least resistant to anoxia, while the rabbit b-wave and the cat a-wave are most resistant to anoxia and least resistant to iodoacetic acid. Azide, on the other hand, affects the rabbit and cat electroretinogram in the same way as iodoacetic affects the rabbit electroretinogram. There is as yet no satisfactory explanation for this discrepancy.

Since formaldehyde, in the concentrations used below, is a specific inhibitor of retinal anerobic glycolysis, it has seemed profitable for the authors to compare its action to that of azide and iodoacetic acid as well as to compare the relative effects of methanol, formate, and formaldehyde on the electroretinogram.

Methods and Materials

The experimental animals were adult rabbits and cats. The animals were anethesized with nembutal, intubated for artificial respiration, and then completely immobilized by curarization. Control experiments had previously established that nembutal in the doses used had negligible effect on the electroretinogram and optic-nerve discharge. Access to the posterior pole of the eye was obtained by removal of the zygoma and portions of the frontal and temporal bones. The electroretinogram was then recorded by means of Ag-AgCl-wick electrodes, one on the cornea, the other on the sclera near the posterior pole. The optic-nerve response, where recorded, was picked up by means of either a steel or tungsten microelectrode, insulated except for the tip, and inserted by visual control into the optic nerve a few millimeters anterior to the chiasm. Access to the intracranial optic nerve was gained by removal of the skull over the frontal and temporal regions and removal of the anterior third of the homolateral hemisphere. Interference with the blood supply to the optic nerve was carefully avoided. Bleeding was controlled by cautery and topical thrombin. A properly prepared experimental animal showed no signs of deterioration five hours after surgical procedures were completed.

The apparatus is described in detail in the appendix.

The stimulus was white tungsten light of one foot candle intensity. The duration of the stimulus was usually 200 milliseconds. Four minutes of dark adaptation were allowed unless the interval between flashes was, of necessity, too short.

Methanol and ethanol were given by stomach tube in the concentration of 25 gm. per liter of water. Formate and formaldehyde were administered intravenously—formate as the sodium salt, neutralized to pH 7.4 in concentrations of 400 mg./cc., and formaldehyde in concentrations of 50 mg./cc.

Results

A. The effect of methanol and ethanol on the electroretinogram

Figure 1 (above) shows the dose of methanol required to affect the electroretinogram in the cat. Two hours after the enteric administration of such a dose, the b-wave is eliminated and a negative electroretinogram obtains. Unoperated animals receiving this quantity of methanol become comatose in a

* It is assumed that the reader is familiar with the conventions regarding the naming of the waves of the electroretinogram and optic-nerve discharge. For information on the naming and derivation of the electroretinogram waves, see Bound's review.[7] The naming of the waves of the optic-nerve discharge follows the nomenclature of Bartley and Bishop.[8]

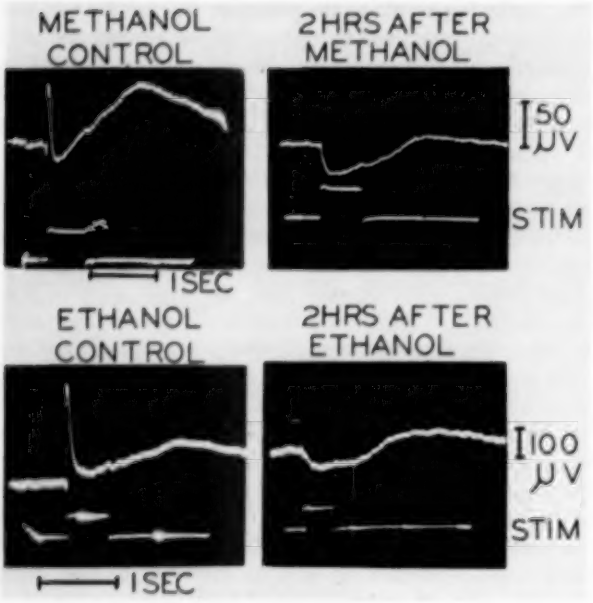

Fig. 1 (Praglin, Spurney and Potts). (Above) The effect of 10 gm./k. of methanol on the cat electroretinogram. (Below) The effect of 15 gm./kg. of methanol on the cat electroretinogram.

few minutes and never regain consciousness. Equimolar doses of ethanol (fig. 1-below) produce identical electroretinogram effects and are similarly fatal. Exactly the same results were obtained with rabbits.

B. THE EFFECT OF FORMATE ON THE ELECTRORETINOGRAM

The dose of sodium formate required to affect the electroretinogram in the cat is 1.0 gm./kg. The results of such an experiment

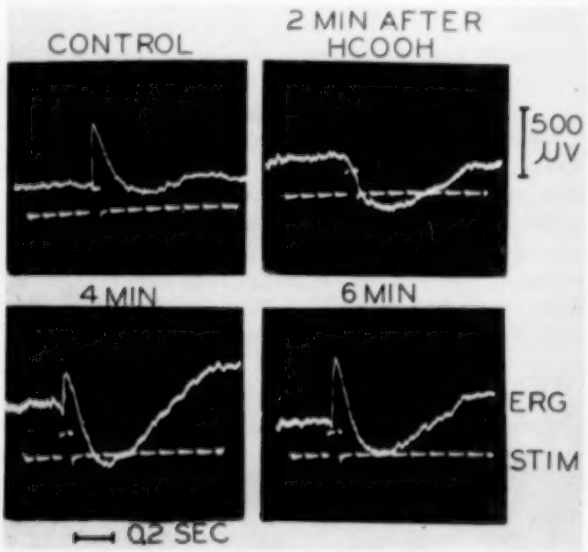

Fig. 2 (Praglin, Spurney and Potts). The effect of 1 gm./kg. of formate on the electroretinogram of the cat.

are shown in Figure 2. As before, the b-wave is eliminated and the electroretinogram consists of only a negative-type electroretinogram. Recovery from this effect occurs within two minutes. It should be noted that the b-wave re-emerges just previous in time to the marked negative deflection caused by formate administration. The dose of formate required for this effect, although large, was below the lethal concentration. It was not possible to produce an electroretinogram effect in the rabbit with any attainable dose of formate.

C. The effect of formaldehyde on the electroretinogram and on optic-nerve discharge

The dose of formaldehyde required to affect the electroretinogram in the cat is 20 mg./kg. The results of such an experiment are shown in Figure 3. The upper tracing shows the electroretinogram, the lower, the optic-nerve discharge with its on and off waves. The immediate effect of formaldehyde is the abolishment of the b-wave and the optic tract discharge. The electroretinogram changes to a large negative-going deflection. Four minutes later, the b-wave has returned to some extent, appearing in the trough of the negative wave. At that time the on-wave of the optic tract reappears. In about 30 minutes a complete return to the control picture is evident. The off-wave of the optic tract returns considerably later in time than the on-wave. In the cat, the reapparance of the off-wave is not correlated with any other event in the electroretinogram optic-nerve discharge.

Figure 4 shows a similar experiment with the rabbit. The control picture shows a typical rabbit ERG and optic-nerve discharge. In the rabbit, the optic-nerve discharge consists of both an early and a late on-discharge as well as an off-discharge. Formaldehyde, as before, immediately eliminates the b-wave. In the optic tract, the late on-wave and off-waves disappear but the early on-wave re-

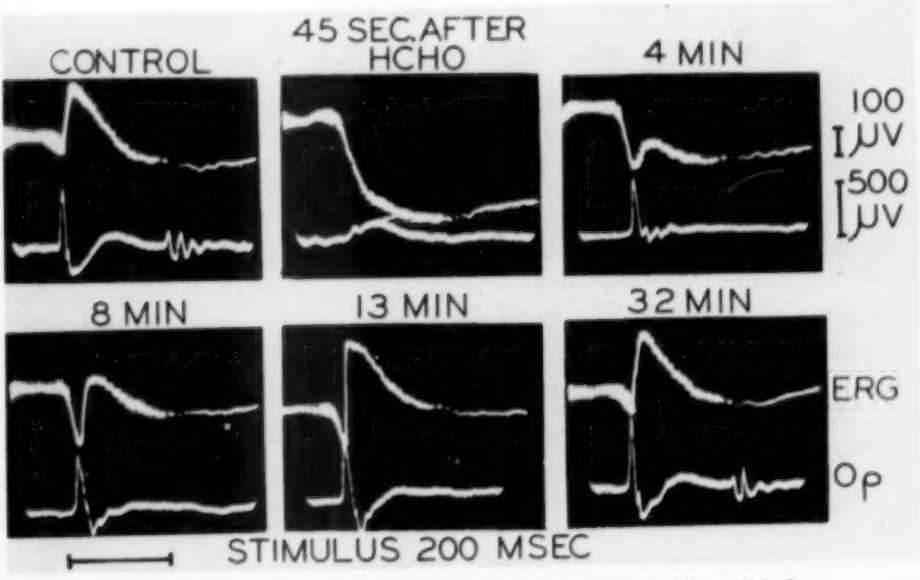

Fig. 3 (Praglin, Spurney and Potts). The effect of 20 mg./kg. of formaldehyde on the cat electroretinogram.

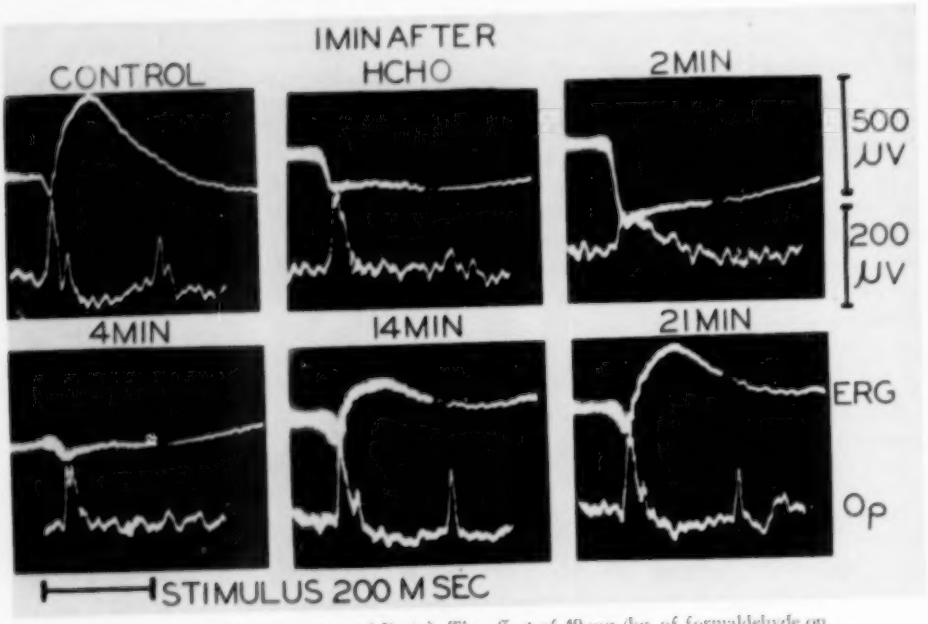

Fig. 4 (Praglin, Spurney and Potts). The effect of 40 mg./kg. of formaldehyde on the rabbit electroretinogram.

mains at first. However, as the negative electroretinogram increases in amplitude, possibly, as Noel suggests, by progressive loss of the b-wave component the early on-wave is in part affected. Recovery is quickly attained and the late on-wave and the off-wave return simultaneously. As in the cat, complete recovery occurs in a few minutes.

In the doses listed here (from 20 to 40 mg./kg.) no whole animal toxic effects were ever noted, and complete recovery of electrical phenomena was always obtained.

D. The effects of formaldehyde and iodoacetic acid

In order to test whether or not formaldehyde and iodoacetic acid act on the retina by the same mechanism, the experiment shown in Figure 5 was performed. A dose of 20 mg./kg. of formaldehyde was given to a rabbit. The usual result, the immediate production of a P-III type electroretinogram, is shown. Two hours were allowed for recovery. At this time 15 mg./kg. of iodoacetic acid was given, and, as with formaldehyde, the b-wave was eliminated. Fifteen minutes after this a second dose of formaldehyde was given. As can be seen, no additional effect could be noted.

Discussion

As stated in the introduction, the results of this investigation yield two types of information—specific data on the relative toxicity of methanol, formate, and formaldehyde and more general information on the components of the electroretinogram and optic-nerve discharge and their interrelationship. These results will be discussed separately.

A. The comparative toxicity of methanol, formaldehyde, and formate

As the experimental results have shown, the effect of all three substances is quite similar—elimination of the b-wave and production or a P-III or negative-type of electroretinogram. The doses necessary for this

TABLE 1

DOSAGES OF DRUGS NECESSARY TO GIVE EFFECT

Substance	Concentration Just Giving Electroretinogram Effect		Concentration Giving 50-percent Inhibition of Retinal Glycolysis[4]	
	Mol./kg. Animal	Relative Concentration Formal. = 1	Mol./L. Homogenate	Relative Concentration Formal. = 1
CH_3OH	0.31	440	3.0	3,000
$HCOO^-$	0.022	32	0.025	25
HCHO	0.0007	1	0.001	1

effect, however, are markedly different, as Table 1 shows.

Only formaldehyde was effective in small enough concentrations for it to be considered as functioning as a specific enzyme inhibitor. There is a striking similarity between the doses of inhibitor required to give the electroretinogram effect and the concentrations required for inhibition of glycolysis reported previously.[4] This correspondence is shown in Table 1. Whatever the significance of the actual concentrations, the correspondence in order of magnitude is striking and cannot be ignored.

The calculations previously made on the basis of the retinal metabolism figures (and on the fact that as little as 10 gm. total dose or 0.005 mols./kg. of methanol can cause blindness in man) apply equally here. The only one of the three substances which is inhibitory to the electroretinogram in concentrations comparable to the human blindness dose is again formaldehyde.

The uniqueness of the formaldehyde effect is underlined by our finding that the two carbon homolog, acetaldehyde, does not disturb the electroretinogram in doses 50 times higher than the effective formaldehyde dose.

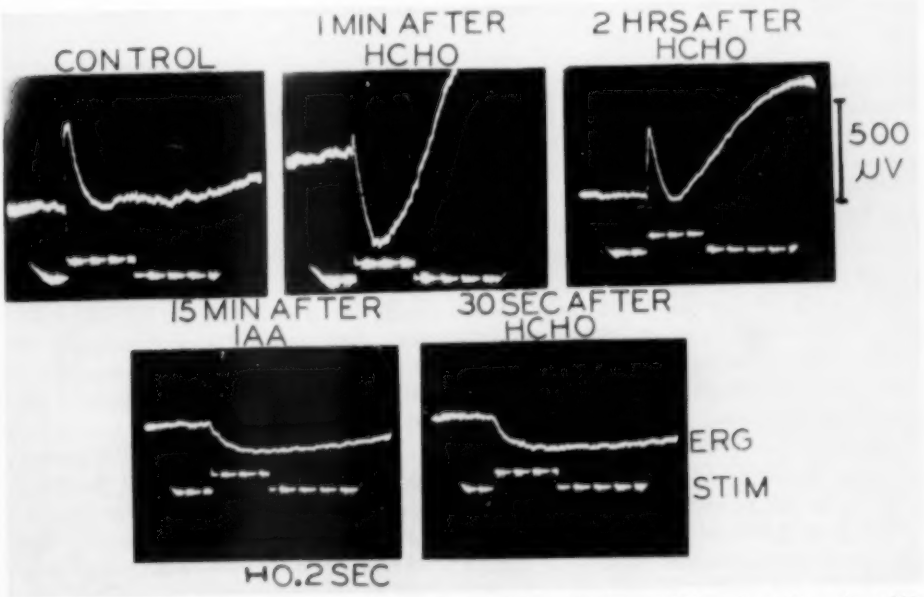

Fig. 5 (Praglin, Spurney and Potts). The effect of formaldehyde and iodoacetic acid on the rabbit electroretinogram. The dose of iodoacetic acid was 15 mg./kg. The dose of formaldehyde in both cases was 20 mg./kg.

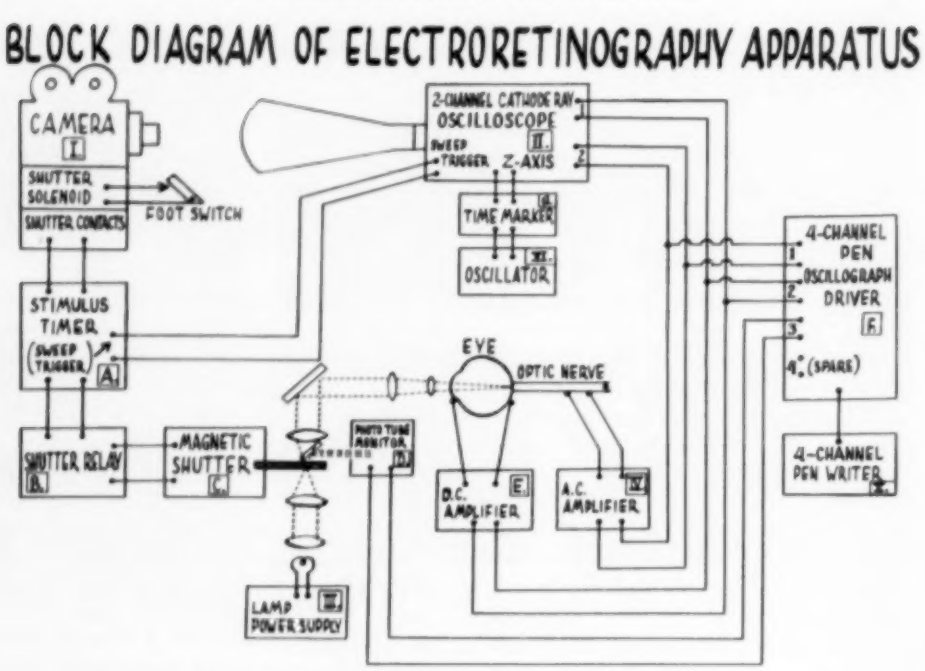

Fig. 6 (Praglin, Spurney and Potts). Block diagram of electroretinography apparatus. Explanation in text.

Conversely, the general nature of the methanol effect is shown by the fact that comparable molar doses of ethanol reproduce the effect exactly—both alcohols requiring 400 times the formaldehyde dose to do this. In addition the alcohols must be given at levels which inevitably produce coma and death; and the conclusion is inescapable that here one is dealing only with the narcosis typical of a large series of organic solvents.

As mentioned earlier, one possible assumption from the foregoing is that formaldehyde is the proximal toxic agent in methanol poisoning and that the difference in susceptibility between primates and other animals is a difference in rate of manufacture of formaldehyde from methanol. In a preliminary attempt to investigate this hypothesis, rabbits were given a daily, intravenous, sublethal dose of formaldehyde (40 mg./kg.) for one to two weeks. Results to date are equivocal, for an occasional animal did appear to have abolition of the electroretinogram. If the differences are based on rate of formaldehyde destruction, of course, such an experiment should be negative in the rabbit and positive in the primate. Work in this direction is being continued.

The effect of formate is intermediate between that of methanol and formaldehyde as shown by Figure 2. Like formaldehyde, a reversible effect is produced at nontoxic levels but the dose required for its action is far in excess of that required by any known metabolic inhibitor. Nevertheless, the action of this substance is reversible and the effect is reached at non-toxic levels. The effects of formate on the electroretinogram are slightly different from those of formaldehyde, moreover, as a comparison of Figures 2 and 3 will show. We may note that the b-wave disappears in both cases but that in the case of

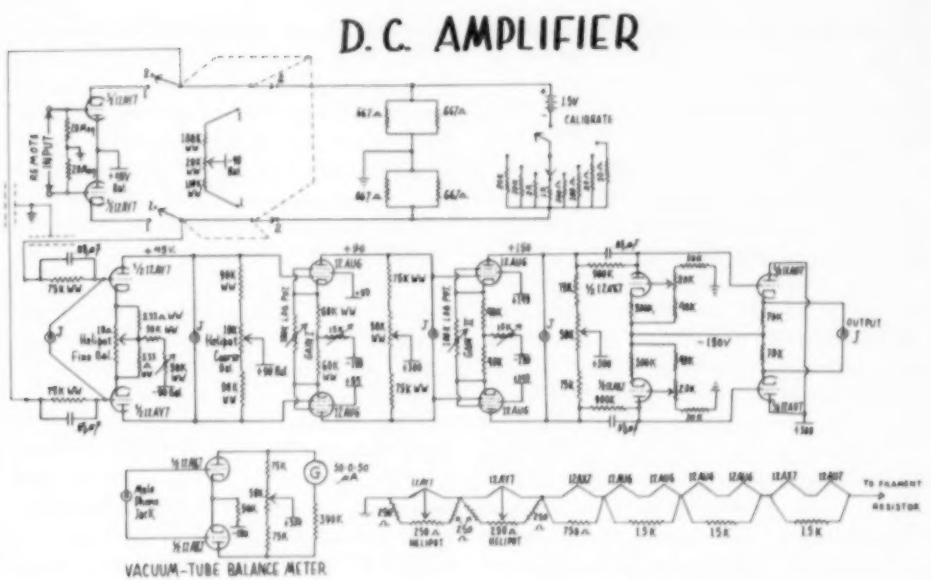

Fig. 7 (Praglin, Spurney and Potts). Schematic diagram of D.C. amplifier for electroretinogram recording.

formate the b-wave reappears in the electroretinogram trace just before the point at which the negative wave descends from the baseline. In the case of formaldehyde, the b-wave reappears in the trough of the negative response. Further investigation of the formate effect will be necessary before it can be decided whether or not formate and

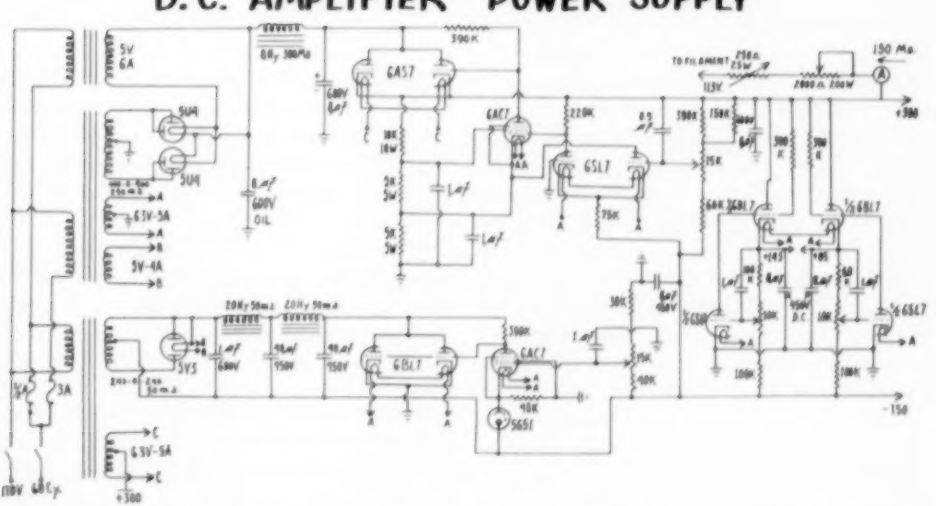

Fig. 8 (Praglin, Spurney and Potts). Schematic diagram of power-supply for D.C. amplifier of Figure 7.

formaldehyde actually differ in their retinal actions.

B. The fractionation of the electroretinogram and optic-nerve discharge by formaldehyde

As we have seen, Figures 3 and 4, which represent, respectively, the effects of formaldehyde on the cat and rabbit electroretinogram and optic-nerve discharge, show essentially the same picture. After injection of formaldehyde, the b-wave is eliminated and taking its place is the classical P-III-type electroretinogram. At the point of greatest negativity, presumably the point at which the b-wave is at its lowest amplitude, electrical activity in the optic nerve is eliminated. During the recovery process, as soon as some return of the b-wave is shown in the electroretinogram, the single on-wave of the cat and the early on-wave of the rabbit reappear. It is not until some time later in both experiments that the remaining waves of the optic-nerve discharge reappear.

The following conclusions can be drawn from these experiments:

1. The on-discharge in the cat and the early on-discharge in the rabbit are probably functionally related to the b-wave, if we can assume, as Noel does, that only when the negativity in the poisoned electroretinogram reaches its maximum is the b-wave completely eliminated. It is, nevertheless, not possible to agree with Granit's theory that the b-wave is the generator of the optic-nerve discharge since Figures 3 and 4 emphatically support Schubert's[9] finding that the on-wave of the optic nerve occurs, in time, previous to the rising phase of the b-wave. This fact is especially well illustrated in Figure 3, the eight-minute tracing. Here, the abnormally large a-wave is exactly synchronized with the on-wave. In view of these facts, it must be considered that, if the on-wave and the b-wave are co-dependent, the b-wave must be thought of as an after-potential to the on-wave, somewhat analogous to spinal cord after-potentials.

2. The a-wave of the electroretinogram occurs synchronously with the earliest wave of the optic tract response. This fact plus the fact that the negative response remains after the b-wave and electrical activity in the optic nerve have been abolished, suggests that the P-III response may have closer connection with receptor excitation than does the b-wave. A few preliminary experiments in this laboratory have lent plausibility to this conjecture by showing that formaldehyde-produced P-III responses are diminished by light adaptation and increased with dark adaptation.

3. The off-waves of the optic nerve and, in the rabbit, the late on-wave can be disassociated from the first on-wave of the optic nerve by formaldehyde. This functional separation is shown very well in Figures 3 and 4. The off-waves always take much longer to recover from the effects of formaldehyde than do the on-waves. The work of Kuffler[10, 11] and Barlow[12] suggest that, while on-discharges in single fibers of the optic nerve may be generally due to more or less direct conduction of impulses from the receptors via the bipolar cells to the ganglion cells, off-discharges occur by means of interaction between parts of the retina on the fringe of the stimulated area with those parts maximally stimulated, via cross-connecting neurons. In the light of this, it is possible that the fractionation of the optic-nerve discharge by formaldehyde is due to a greater susceptibility of retinal cross-connections than of the direct transmission chain to formaldehyde.

4. In the rabbit the late on-wave and off-wave seem to be related. This conclusion is reached since on formaldehyde poisoning they disappear together and reappear together.

C. A comparison of formaldehyde, iodoacetic acid, and azide

A comparison of our results on formaldehyde with those of Noel on iodoacetic acid and azide yields the following information:

1. The actions of azide and formaldehyde on the electroretinogram are identical. Both act in a completely reversible fashion.

2. Whereas formaldehyde reversibly fractionates the optic-nerve discharge, azide causes a lasting depression of optic nerve excitability.

3. Both in the rabbit and in the cat the action of iodoacetic acid is irreversible.

4. In the rabbit the initial effects of iodoacetic acid resemble those of azide and formaldehyde.

5. In the cat Noel claims that the b-wave is more resistant to iodoacetic acid than the a-wave. This effect differs from that of formaldehyde and azide where the opposite is true.

Although these substances have in common the property of inhibiting glycolysis at various stages, one cannot explain the divergence of their late effects with the help of known biochemical mechanisms.

This much is certain: When a rabbit (fig. 5) is demonstrated to react to formaldehyde, allowed to recover, and given iodoacetic acid (15 mg./kg.) to abolish the b-wave, a second dose of 20 mg./kg. of formaldehyde does not produce its characteristic effect.

Summary

1. The relative toxicities of methanol, formate, and formaldehyde on the retina of experimental animals have been investigated by electrophysiologic techniques. The doses of these substances necessary to abolish the b-wave of the electroretinogram were:

Formaldehyde, 0.0007 mol./kg. of animal; formate 0.025 mol./kg.; and methanol 0.03 mol./kg. This result closely parallels the findings previously reported for inhibition of glycolysis in retinal homogenates. A dose of methanol sufficient to cause human blindness amounts to 0.005 mol./kg. Therefore, only if the methanol were converted to formaldehyde would this concentration of methanol be sufficient to cause poisoning. This is considered additional evidence that formaldehyde is the proximal toxic agent in methanol poisoning.

2. It has been shown that formaldehyde is a potent but reversible fractionating agent for both the electroretinogram and optic-nerve discharge.

3. Time relations in our records support Schubert's conclusion[9] that the b-wave cannot be considered the generator of the optic-nerve discharge.

4. Fractionation of the optic-nerve discharge by formaldehyde demonstrates the on- and off-waves of the optic tract arise by different mechanisms.

University Hospitals (6).

Appendix

Description of experimental arrangement

The text refers to the block diagram of the experimental setup shown in Figure 6.

The subject is placed in a light-tight, shielded, ventilated room which occupies a third of the laboratory. The light source is an 18 A. 6 V. compact filament projection bulb or an electronic flash bulb (G.E. FT 524). The tungsten lamp is powered by an ATR #620-C-EL 10 battery eliminator (III). Low ripple and stability is assured by the connection of a lead storage battery across the lamp terminals in parallel with the power supply. The photoflash bulb is powered by a 300 watt, 5,000 volt supply. Included in this supply is a thyratron pulsing circuit for firing the lamp. Repetition rates up to 1,000 flashes per second are possible with this device.

The duration and frequency of the stimulus is controlled in the case of the tungsten bulb by an electromagnetic shutter (C) which is driven by relay (B) and controlled by timing circuit (A).

The timing circuit is capable of recurrent operation in the range 0.05 to 1,000 cycles per second or it may be externally triggered for single stimulus operation. In routine experimental procedure, depression of foot switch opens shutter of camera (I), the flash contact of the shutter closes a relay which initiates the stimulus timer. Immediately on closing of the switch, the sweep of the dual beam oscilloscope (II) (Dumont #322, 5SP11 long persistence screen) is triggered by the timer. At the same time, a variable delay multivibrator circuit which times a delay variable from 100 microseconds to six seconds, is triggered. At the end of this delay, when the oscilloscope sweep has progressed sufficiently to establish a zero baseline, the stimulus is initiated by the triggering of a second multivibrator which accurately times the stimulus duration in a range of 10 milliseconds to 10 seconds by

actuating a fast-acting telephone-type relay. In turn, this relay energizes the electromagnetic shutter which consists of a rotary solenoid (#8002, Price Electric Co.), modified by the addition of bearings and a lever arm, for interrupting the light beam.

The intensity of light is modified by Wratten neutral density filters and the color selected by interference filters placed in the light path. With this arrangement, light intensity and wave length can be varied through the entire physiologic range. A phototube monitor (D) amplifies and records the duration and intensity of the light stimulus.

The electroretinogram (ERG) is picked up by nonpolarizable Ag-AgCl-wick electrodes placed on the cornea and posterior pole of the eye. The optic-nerve discharge is recorded intracranially between a steel or tungsten microelectrode placed in the optic nerve anterior to the chiasm and the posterior electroretinogram electrode. An indifferent electrode, common to both sets of electrodes, is placed in the neck region.

The electroretinogram is amplified by a D.C. amplifier (E) and the optic nerve discharge by a Grass model P4 capacity-coupled amplifier (IV).

Since a D.C. amplifier of low drift is essential for completely accurate recording of the electroretinogram, a circuit diagram of the D.C. amplifier with its associated power supply in shown in Figures 7 and 8. After a warm-up period of two hours, the drift, with the input shorted, is less than 300 microvolts per hours.

A sine-wave oscillator (Hewlett-Packart #200D) is used to trigger time marker (G) in the range of seven to 70,000 cycles per second. The sine wave is converted into a trigger pulse which fires a multivibrator circuit once each sine wave cycle. The square wave produced by the multivibrator circuit is applied to the z-axis input of the oscilloscope and produces an intensity-modulated time signal on the oscilloscope trace. The width of the time marker is variable from 10 microseconds to 10 seconds. An internal phase-shift oscillator supplies additional time base frequencies of one and five cycles per second.

The outputs from the amplifiers may be displayed and recorded by the dual beam oscilloscope (II) or the pen-writer (V) (Grass 4 channel pen unit).

The authors are indebted to Dr. H. K. Hartline for certain suggestions regarding the experimental set-up.

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STUDIES ON THE VISUAL TOXICITY OF METHANOL*

V. The role of acidosis in experimental methanol poisoning

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Acidosis is prominent among the clinical signs in human methanol poisoning. Røe[1] postulates that, "severe acidosis is necessary for the development of amblyopia and amaurosis." Benton and Calhoun[2] state, "the acidosis does not appear to be the cause of amblyopia but it does act as an accelerating force. If acidosis can be corrected before permanent ocular damage has resulted, a return of normal visual acuity can be expected." The standard therapy of methanol poisoning today consists of combatting acidosis.

In view of this, we have set ourselves the goal of trying to learn with laboratory animals whether acidosis is the cause, or the companion, of visual loss in methanol poisoning. Immediately the question arises as to whether any animals other than humans develop acidosis from methanol. The literature apparently provides both yes and no answers. The second question is whether methanol causes visual damage in nonhuman species. Again the answers in the literature are conflicting. Our work in attempting to solve the problem of whether methanol causes acidosis or visual damage in nonhuman species comprises the material for this paper.

Literature

I. Acidosis

In 1912, Schmiedeberg[3] first postulated the development of acidosis in methanol poisoning. Citing Pohl[4] and Bongers,[5] who had studied the metabolism of methanol in experimental animals, Schmiedeberg considered that in these animals the resultant formic acid was neutralized either by blood base or by newly formed ammonia. Schmiedeberg was undecided on the role of acidosis in visual loss.

Król (1913),[6] acting upon Schmiedeberg's theories, investigated the ammonia output in the urine of three dogs given nonlethal divided doses of methanol. In all three he found the amounts of urinary ammonia were doubled to quadrupled. In one dog he also determined urine formates, finding that formates neutralized only a quarter of the ammonia. Król did not learn what acid or acids did neutralize the remaining 75 percent of the ammonia, although it was not oxalic acid. However, he believed the increased ammonia formation meant his dogs were acidotic.

Grignolo (1913)[7] determined the hydrogen ion concentration of aqueous and blood serum in three dogs with the concentration apparatus of C. Foa. Although he found a very slightly higher hydrogen-ion concentration after single sublethal doses of methanol, Grignolo concluded that these were "changes which do not depart significantly from the physiological values." In spite of this conclusion, he has been erroneously quoted in the literature[8] as having demonstrated acidosis in dogs and as believing the increased hydrogen ion concentration parallelled morphologic changes in the eye tissues.

Tyson and Schoenberg[8–10] reported that methanol produced acidity of the aqueous in dogs and rabbits and acidosis in dogs. The authors presented no data about normal controls. The statement about acidosis was

* From the Eye Service, Department of Surgery, University Hospitals, and the Laboratory for Research in Ophthalmology, Western Reserve University. This work was aided by a grant from the Office of Naval Research. A preliminary report on this material was given before the East Central Section of the Association for Research in Ophthalmology, Pittsburgh, Pennsylvania, January, 1954.

based on determinations of blood electroconductivity with a resistance bridge.

They stated that an increase in electroconductivity of the blood might result from the breaking up of the corpuscles, from an increase in the hydrogen ion content, or from any increase in the inorganic salts. Of these three possibilities, they decided that the increased electroconductivity of their dogs' blood was due to an increase in the hydrogen ion concentration because blood serum following methanol was acid to phenolphthalein.

These authors did not realize that blood of normal dogs is acid to phenolphthalein.[11] These strictures also apply to their description of an acid aqueous.[12] An additional source of error was that their animals suffered severe anoxic anoxia from unventilated inhalation experiments. Severe and prolonged anoxic anoxia causes acidosis (Van Liere).[13] Koehler, Brunquist and Loevenhart[14] found CO_2-combining capacity dropping to 9.8 vol. percent in pigs with anoxic anoxia. It is evident that the conclusions of Tyson and Schoenberg that methanol poisoning in experimental animals produced acidosis has no justification on the basis of their experimental data.

Haskell, Hileman, and Gardner (1921),[15] using the gasometric apparatus of Van Slyke and Cullen to determine plasma CO_2-combining capacity in dogs, reached these conclusions:

"In dogs poisoned with methanol, the severity of the intoxication is, at times, entirely at variance with the degree of acidosis. . . .

"Alkali, in the form of sodium bicarbonate, has, in itself, little or no influence on the course of poisoning."

Although the authors evidently used large numbers of dogs, they give data on CO_2-combining capacity for only three: two receiving single oral doses of 7.9 gm./kg. had normal CO_2-combining capacities shortly before and after death; one receiving 6.3 gm./kg. survived with the following CO_2-combining capacities: 43.9 vol. percent before methanol, 33.2 vol. percent at 24 hours, and 29.6 vol. percent on the third day. The authors reported seven dogs given 6.3 gm. methanol/kg., orally, plus 1.25 to 5.0 gm. $NaHCO_3$/kg./24 hours given either orally or intravenously. All except one died and at intervals bearing no relationship to the amount of sodium bicarbonate. No CO_2-combining capacities were reported as being done on this treated series.

Loewy and Münzer (1923)[16] disagreed with Król's conclusion that increased urinary ammonia formation meant that the dogs were acidotic. In two rabbits and one dog, they found no decrease in the CO_2-combining capacity or increase in pH. Their use of usually sublethal, and often subtoxic, doses of methanol, their paucity of experimental animals, and their lack of duplicate determinations makes their work inconclusive, however.

Leo (1925)[17] next tackled the problem of experimental acidosis by means of survivorship with and without therapy with sodium bicarbonate. He made no CO_2-combining capacity determinations and used four dogs. Two dogs received 1.45 gm. methanol/kg. every day for six days, by stomach tube, a low dosage. One of these two dogs received an average of 2.5 gm. $NaHCO_3$ every day, seven times. The control dog died on the eighth day, whereas the treated dog was completely well at that time. No experimental data were given for the second pair of dogs except that the control dog died on the sixth day and the treated dog on the 10th day. Leo stated that $NaHCO_3$ was without effect in the therapy of methanol-poisoned mice, rats, and rabbits, but gave no experimental data. This is not conclusive evidence, nor does Leo make such a claim. The German dog shortage unfortunately prevented his doing further work. He believed that these experiments indicated a species difference in reaction to methanol, and that sodium bicarbonate successfully combats methanol-induced acidosis in dogs.

Von Oettingen[18] believed that Leo's assumptions were supported by the work of Rewiger (1922).[19] Rewiger gave six dogs single, subtoxic, oral doses of methanol (1.04 to 1.7 gm./kg.) and then determined their urinary nitrogen output by the Kjeldahl method. Following methanol he found maximal increases in the daily urinary nitrogen of from 1.3 to 1.7 times their normal output. He likewise gave two rats oral methanol at comparably subtoxic levels for the species (3.8 and 4.4 gm./kg.) and found no effect on the urinary nitrogen. Rewiger subscribed to the theory that there is a parallel between eye damage and altered protein metabolism, but reported no eye examinations in his animals.

Clark and Gibson (1933)[20] reported that in dogs, while "sodium bicarbonate in sufficient amounts to maintain a normal acid-base balance was ineffective as was the repeated administration of glucose alone, . . . a combined therapy with sodium bicarbonate seems successful." Experimental details were not published in this brief summary of an orally presented paper, but Dr. Clark very kindly made the experimental protocols available to us.[21] Doing CO_2-combining capacity by the Van Slyke method, Clark found that, of four dogs fatally poisoned with orally administered, repeated doses of methanol, three developed severe acidosis.

1. From a normal of 41 vol. percent to a low of 20 vol. percent.
2. From a normal of 41 vol. percent to a low of 14 vol. percent.
3. From a normal of 45 vol. percent to a low of 11 vol. percent.

The fourth dog died before a postmethanol blood sample was obtained. Only one dog was given a single oral dose. This dog survived 7.0 gm. of 20 to 25 percent methanol/kg. without developing acidosis (from a normal of 36 vol. percent to a low of 31 vol. percent). Methanol doses and doses of therapeutic substances varied in each experiment. However, even although dosages cannot be compared, the increased survival rate of the group treated with sodium bicarbonate and glucose and insulin was not mathematically significant. Survivals were as follows:

1. Methanol only—one out of five survived.
2. Methanol and glucose—one out of three survived.
3. Methanol and $NaHCO_3$—none out of three survived.
4. Methanol and $NaHCO_3$ and insulin and glucose—four out of six survived.

Røe (1948)[1] measured the alkali reserve in rats and rabbits. Nine rats were given single doses of 6.3 gm. methanol/kg. by stomach tube. Our rat LD_{50} per os was 9.5 gm./kg. (50-percent solution) and that of Alder, Buschke, and Gordonoff[22] (LD_{60}) was 8.3 gm./kg. (70-percent solution). Thus Røe's dosage is evidently sublethal for rats. He collected bloods by decapitation on the two following days, finding all CO_2-combining capacities lying between 47 and 60 vol. percent. He also did a series of five rabbits. Two received daily oral doses of methanol varying from 2.4 to 5.5 gm./kg. for three and seven days. Neither showed acidosis. Three received single doses (6.3 and 7.9 gm./kg.). Of these, one of the two 6.3 gm. rabbits showed a drop in CO_2-combining capacity of 20 vol. percent on the second day, returning to normal on the third. This is not unusual for rabbits (see later). Values prior to administration of methanol were not reported for the other two, but second-day CO_2-combining capacities were normal. Røe concluded that "like the rats, the rabbits showed no signs of acidosis."

Because of its bearing on aspects of our experimental work,[23] a description of two early works on human acidosis is indicated. The first clinical use of alkali therapy was made by Harrop and Benedict (1920)[24] who investigated their patient's alkali reserve because of the work of Schmeideberg[5] and Król.[6] Their patient drank about 2.5 to 3.0 gm. methanol/kg. in one evening. About 48 hours later she was almost blind and was acidotic. $NaHCO_3$ therapy corrected the acidosis. On the second day after ingestion

of methanol, the patient was found to have 2,200 cc./liter of N/10 titratable organic acids in her urine. This dropped four days later to a normal 200 to 400 cc. N/10 acid/l. The method used for the determination of titratable organic acids was that described by Van Slyke and Palmer (1919).[28] Later in 1920 Van Slyke and Palmer[36] also described a patient who survived ingestion of methanol and who also showed an increase in titratable organic acids in his urine. Neither Harrop and Benedict nor Van Slyke and Palmer were able to ascertain what specific acid caused the increase following methanol. It was not caused by lactic, formic, or aceto-acetic acid.

II. Ocular effects

The production of clinical ocular damage by methanol in nonhuman animals has been reported by some authors and denied by others. Likewise, there is disagreement about the production of histologic changes in experimental animals. A critical discussion of the much-debated questions relating to histopathologic changes in the peripheral visual apparatus is beyond the scope of the present report; however, for the sake of completeness, we are reporting the authors' conclusions on histologic experiments.

In humans, methanol frequently causes ocular signs and symptoms and blindness, about the appearance of which there is essential agreement in the literature.[2, 27, 29] It is for these effects that we must look in the papers on experimental poisoning. Four common sources of confusion should be considered before discussing the individual papers:

One error is the conclusion that animals in the early stage of intoxication (the first few hours after administration of methanol) who bump or stumble over objects are blind. These are unquestionably ataxic manifestations of alcoholic intoxication and are dependent upon temporary alterations of higher cerebral functions rather than due to any impairment in function of the peripheral visual apparatus. One does not observe blindness at this stage in human cases.

A second error is that visual impairment following exposure keratitis is due to a specific ocular effect of methanol. All authors are agreed that nonprimates shortly after a sufficiently large dose of methanol become comatose. During coma, which can last as long as four days, the eyelids are usually open. A severe exposure keratitis with secondary bacterial invasion occurs in these cases and results in corneal opacification, which undoubtedly diminishes vision. This nonspecific secondary damage is not comparable to the methanol blindness of humans.

The third error is concerned with altered pupillary sizes and reactions. These changes are common in all nonprimates; but neither in the literature nor in our experience do they show correlation with ocular damage as evinced by ophthalmoscopic and histopathologic appearances. They are associated with semicomatose and comatose states. We believe that in nonprimates the mechanism of production of these pupillary changes is one common to all anesthetic agents and is unrelated to the specific ocular damage caused by methanol. Nystagmus is probably developed on a similar basis since higher alcohols are also reported to cause it; however, no authors have claimed loss of vision because the animals developed nystagmus.

A fourth error is that comatose and moribund animals who do not respond to visual stimuli are blind from the toxic amblyopia of methanol. Animals in such states do not respond to *any* type of stimulus.

In 1896 Joffroy and Serveaux,[29] in a study of acute intravenous and intramuscular methanol poisoning, reported nystagmus and pupillary changes (mydriasis and miosis) in dogs and rabbits. In two chronically poisoned dogs no eye changes were noted. These authors did not report having done ophthalmoscopic examinations.

Baer (1898)[30] gave rabbits single oral doses of methanol. He described early pu-

pillary changes; with lethal doses he often found nystagmus.

Ward Holden (1899)[31] was the first worker to claim that he produced the toxic amblyopia of methanol poisoning in experimental animals. This claim was based upon one dog who received 4.0 gm. methanol/kg. by stomach tube on days one and five of the experiment. Holden described early pupillary changes and blind drunkenness. On day seven he reported ocular irritation as shown by the dog's rubbing his eyes with his paws. On day 15 (10 days after the last methanol) the corneas became diffusely hazy. On day 16 the dog was found dead. The autolyzed eyes were sectioned and were said to show retinal ganglion cell and optic nerve degeneration caused by methanol. An autopsy to determine the cause of death was not reported.

Harry Friedenwald (1902),[32] in an abstract of an oral presentation, reported ganglion cell degeneration in rabbits with chronic methanol poisoning. No experimental details were published about clinical eye examinations.

Birch-Hirschfeld in a series of scholarly papers (1900, 1901, 1902)[33–35] reported his experiments in detail. He poisoned seven rabbits, three hens, four dogs, and three rhesus macaques with repeated oral doses of methanol. He described primary ganglion cell degeneration of the retina occasionally followed by optic-nerve degeneration. His clinical observations are of considerable interest. All of his animals with the exception of the hens received almost daily ophthalmoscopic examinations and tests for objective signs of visual defects. Of these animals only one, a monkey, was considered by Birch-Hirschfeld to have definite evidence of ocular damage. This monkey, given methanol for 15 days (amounts of methanol/body weight were not reported but produced toxic symptoms), showed from the 11th experimental day optic atrophy and dilatation and tortuosity of the retinal veins along with behavior indicating possible visual loss. One dog, poisoned for 35 days, showed on one examination a transient hyperemia of the discs with dilatation of the retinal veins, but had no clinical evidence of visual loss. Only in the monkey did Birch-Hirschfeld consider the clinical presence of a toxic amblyopia a certainty.

Reid Hunt (1902)[36] reported experiments on rabbits and dogs in which he believed one dog became blind. This dog, who was fatally poisoned with repeated doses and who had repeated episodes of unconsciousness, developed a mucopurulent conjunctivitis followed by corneal clouding. When the corneas became blue, blindness was observed. This case has been extensively cited[18] as methanol toxic amblyopia in dogs. The description in the paper is of blindness due to exposure keratitis and not to methanol toxic amblyopia.

Lesieur (1906),[37] giving lethal intravenous doses of methanol and other alcohols to rabbits, reported all alcohols caused nystagmus rarely and mydriasis frequently.

Nicloux and Placet (1912)[38] reported dilated pupils in one dog fatally poisoned with intravenous methanol.

The paper by Igersheimer and Verzár (1913)[39] has been misquoted[18] in that it is cited as showing methanol amblyopia in hens. Igersheimer and Verzár reported that hens showed "diminishing of the light sense with methanol (that is, weaker scratching [for food] with diminishing light)." The authors said, "This raises the question whether these light sense findings are indicative of changes of the retina itself or should they be interpreted as cerebral fatigue symptoms. We cannot venture at this time to give the answer to this question on the grounds of our experiments." They also found no definite histopathologic changes in the eyes of their experimental animals.

Król (1913)[8] stated that none of his three dogs nonfatally poisoned with divided doses showed any ocular damage. No ophthalmoscopic examinations were reported. Król's doses were barely toxic and well below the

approximate oral minimum lethal dose for dogs.

Kasass (1913)[40] gave 40 rabbits toxic oral doses of methanol for periods varying from one to 267 days. On pathologic examination he found "changes in the vascular membrane, in the membranes of the optic nerve, in the retina, beginning with dropsy and degeneration up to albuminuric retinitis, and, in the optic nerve beginning with parenchymatous degenerated neuritis up to axial atrophy."

In 15 of the 40 rabbits, Kasass reported ophthalmoscopic changes, the most frequent of which (14 rabbits) consisted of narrow retinal arteries. Dilated retinal veins occurred in four. Pale discs "which did not have any special significance" occurred in eight. "White discs of suspicious appearance" were seen in three and acute optic atrophy in three. Kasass was unable to devise any test with which he could determine the presence or absence of vision in rabbits.

Evaluation of this paper is difficult. In the early portion, Kasass stated that "numbers 17, 21, and 23 have to be excluded since they died from other causes"; yet he described the pathologic changes in the peripheral visual apparatus of these three rabbits and based his conclusions on findings in these three animals as well as in others. The unique difficulty of a funduscopic diagnosis of optic atrophy in the presence of the myelinated nervehead of the rabbit needs no emphasis.

Langgaard (1913)[41] saw nystagmus in one of a series of rabbits fatally poisoned with oral methanol.

Tyson and Schoenberg[9,10] reported acute and chronic inhalation experiments using five guinea pigs, one rabbit, nine dogs, and one monkey. They reported ophthalmoscopic changes in 100 percent of the examined dogs and the monkey, and retinal ganglion-cell degeneration in all animals. In passing, it should be mentioned that, except in two instances, all pathologic material was obtained from animals dead for an unknown period of time. Fundus changes reported in the dogs consisted, with one exception, of hyperemia and edema of the optic discs, and dilatation and darkening of the retinal veins.

Their dogs were placed in an unventilated box. Calculations show severe anoxia was produced. The authors made ophthalmoscopic examinations immediately after removing the dogs from the box; and eye changes were *never* reported after the few times in which the authors stated that free ventilation had been given.

Anoxia is reported to cause dilatation and darkening of the retinal vessels, especially the veins (Cusick, Benson, and Boothby;[42] Duguet, Dumont, and Bailliart[43]), enlargement of the blind spot (Goldmann and Schubert[44]), and is believed "to play a role in the production of visual defects associated with papilledema as sometimes occurs in hemorrhagic states" (Walsh[45]).

Thus, anoxia alone can cause the ocular findings Tyson and Schoenberg ascribed to methanol. These eye changes are found in uncomplicated anoxia. Tyson and Schoenberg's unventilated box provided increasing carbon dioxide in addition to decreasing oxygen amounts. It is unnecessary to consider toxic effects of carbon dioxide here.

The exception in the fundus changes was in one dog who from eight to 50 days had optic discs which were "paler than in the normal dog examined." The monkey apparently received free ventilation during at least most of the experiments, as was frequently noted by the authors. On day 19, the discs were considered hyperemic as compared with the first examination; this was subsequently not remarked upon, the monkey dying at 22 days. This might represent toxic amblyopia; on the other hand, they report one dog who was killed when the assistant accidentally closed the vents for too long a time.

In 1920, the official protocol of a meeting[46] stated that, in association with the report of human cases of methanol poisoning, "Birch-Hirschfeld presented data of retina and

optic nerve pathology in experimental animals." Apparently this was merely a summary of his 1900 to 1902 experiments, a surmise abetted by the fact that Schwarzkopf[47] working under Birch-Hirschfeld in 1922 failed to cite any work more recent than 1902.

Schanz (1920)[48] claimed that exposure of the eye to light precipitated the toxic amblyopia of methanol poisoning. Schanz gave three rabbits single sublethal oral doses of methanol. One eye of each rabbit was covered, except during ophthalmoscopic examinations. One rabbit killed after 10 days showed no abnormalities, O.U. Rabbit No. 2, killed after 19 days, had a normal unlighted eye and large exudates in the lower half of the retina of the lighted eye. Schanz gave no description of these exudates, but this does not resemble the human toxic amblyopia 19 days after methanol poisoning. No experimental details whatsoever about the third rabbit were reported. See discussion of this paper by Schwarzkopf.[47]

Friedenwald and Felty (1920)[49] gave rats, mice, guinea pigs, rabbits, and dogs methanol doses comparable to those given by Birch-Hirschfeld. Ophthalmoscopic examinations were not done. They found that Birch-Hirschfeld's histologic findings could be explained by artefacts due to fixation techniques rather than to methanol poisoning.

Bills and Maukin (1921)[50] exposed white rats to methanol fumes, getting toxic systemic effects and even death. However, no decrease in brightness sensitivity was observed.

Schwarzkopf (1922)[47] chronically poisoned three rabbits and two dogs with methanol via stomach tube. He found retinal ganglion cell degeneration and occasional optic nerve degeneration but no definite clinical or ophthalmoscopic evidences of ocular damage. His experiments also dealt with exposure of the eyes to light, which he reported to be without effect on methanol poisoning.

deSchweinitz (1923)[51] reported data on three chronically poisoned dogs studied by himself and co-workers. From oral doses given every two to three days for nine to 80 days, the dogs showed marked intoxication but "during life gave no indication, ophthalmoscopic or otherwise, of defective vision" and pathologic examinations failed to show retinal ganglion cell degeneration. In one dog there was very slight veiling of the discs on one day, but deSchweinitz did not consider this significant.

Munch and Schwartze (1925)[52] studied acute oral toxicities in rabbits. Their only reported eye finding was frequently occurring nystagmus.

Rost and Braun (1926)[53] concluded, on the basis of the literature as well as of their own work, that the specific poisoning by methanol of the nervous apparatus of human eyes was also found in animals. Their conclusions were based upon the following evidence. In poisonings of dogs, rabbits, hens, ducks, and a cat by orally administered, divided doses, one dog after five days of deep narcosis was found to have clinical eye changes. This was a blue-white opacity of the cornea. Ganglion cell changes were found in specimens obtained after dogs had been dead for unknown times; but no pathologic changes were found in the eyes of dogs experimentally killed. Rost and Braun also reported nystagmus in rabbits.

Alfred Leo (1927)[54] gave single doses of methanol to four dogs and chronically poisoned two dogs (both orally). In all experiments he found no eye damage or changes except one episode each in two dogs of early pupillary changes. Leo did not do ophthalmoscopic or histopathologic examinations of the eyes.

Weese (1928)[55] used mice in chronic inhalation experiments. Groll, who examined the histologic sections of the eyes, found degenerative changes of the nervous elements of the retina but was of the opinion that these changes were not necessarily the result of an intravital process.

Noè (1929),[56] on the basis of acute intravenous poisoning experiments, claimed that

rabbits sometimes became blind. The evidence upon which this statement of blindness was made consisted of two rabbits who (1) did not raise their heads to a bright light in a dark chamber and (2) did not immediately go to a proffered cabbage leaf. One of these rabbits was moribund at the time of testing, dying less than four hours later. In consideration of the unpredictability of normal rabbit behavior, we do not find this evidence convincing. Noè reported no ophthalmoscopic or other ocular findings.

Keeser (1931, 1931)[57, 58] found formaldehyde in the vitreous of rabbits given repeated sublethal doses of methanol. After incubating surviving calves' vitreous with methanol, he also found formaldehyde, which he considered the toxic agent in methanol poisoning. He reported, without giving the numbers of rabbits used, that "the animals, which for two weeks had daily received 3.0 cc. methanol plus 0.5 gm. ammonium carbonate/kg. body weight in dilute aqueous solution, showed less extensive changes in their organs by macroscopic examination than those animals who were given only methanol." Keeser did not specify what organs or what changes.

McCord (1931)[59] and Scott, Helz, and McCord (1933)[60] gave single and repeated doses of methanol by skin absorption, inhalation, and ingestion to rats, rabbits, and rhesus monkeys. Reported clinical ocular findings were: early pupillary dilatation and slow reaction to light (species unidentified), corneal opacification in some of the rats and rabbits, clinical optic atrophy in rabbits, and blindness (one monkey, other species unidentified). No correlation was given between dosage and clinical ocular findings. They reported the following histopathologic changes: parenchymatous degeneration and focal necrosis of the liver; parenchymatous degeneration of the epithelium lining the convoluted tubules of the kidney; increased blood-forming activity of the spleen; edema, congestion, and desquamation of the alveolar epithelium and pneumonic consolidation of the lungs; edema, granular degeneration, and necrosis of the muscular fibers of the heart; frequent hyperplasia of the lymph nodes; capillary congestion, edema, and patchy degeneration in the neurones of the spinal cord and brain; peripheral neuritis; constant retinal changes consisting of marked congestion of the choroidal vessels, edema, patchy degeneration of the ganglion cells; and rarely, including one monkey blind at death, degeneration of the optic nerve. The report that a monkey was blind at death is significant. Unfortunately protocols giving details of the clinical and histopathologic findings are no longer available.

Sammartino (1933)[61] gave a "series" of one rabbit a single intravenous sublethal dose of methanol. His only abnormal ocular finding was transient "hyperemia of the papillary veins" in the fundi. In six rabbits given formaldehyde and five given formic acid, he found fundi always normal. A minor fundus change of a debatable nature in one of a species with unusual discs does not constitute toxic amblyopia in our opinion.

Harada (1937)[62] studied the antihelminic action of methanol and nine other drugs in mice and dogs. Along with four other drugs in dogs, methanol was reported to cause "sight damage, as was shown by blindness, mydriasis, slow pupillary reaction to light, anisocoria, and often fixed pupils." Harada gave no evidence as to how he determined "blindness"; apparently his conclusion of blindness was based on the early pupillary changes in narcotized animals. Harada described a variety of histologic degenerative changes common to all 10 drugs; no details of techniques were given.

Alder, Buschke, and Gordonoff[22] worked with rats and rabbits giving both oral methanol. White rats were fatally poisoned with single and repeated doses. Their eyes showed no ophthalmoscopic changes, and with respect to histopathologic findings, the authors concluded that any alterations could equally well have been caused by artificial

means. Although white rabbits nonfatally poisoned with repeated doses showed no ophthalmoscopic changes, their retinas showed ganglion-cell degeneration. These rabbits were given 1.4 gm. of a 70-percent solution of methanol/kg. on days 1, 3, and 4. For the rabbit this dosage should be subtoxic; in our hands a single oral dose of 7.0 gm. of 30-percent methanol/kg. was the approximate LD_{100}.

Tomita (1939)[63] fed dogs repeated daily doses of 1.4 to 12 gm./kg. of 30-percent methanol mixed with cow's milk until death occurred. The diagnosis in some of these dogs of clinical visual damage was based upon pupillary changes or corneal opacification following exposure keratitis. Ophthalmoscopic examinations made on some of the dogs always showed normal eyegrounds except for one dog poisoned for 224 days and showing engorged retinal veins toward the end. Tomita reported degenerative changes of the ganglion cells of the retina; degeneration of the optic-nerve fibers; softening, endarteritis, and bleeding in the brain. Fixation of tissues was done from zero to six hours after death.

Koppanyi and Cutting (1941)[64] found no blindness in two dogs for several weeks after recovering from single oral doses of 16 gm./kg. of absolute methanol, and in one dog given 8.0 gm./kg. All three were treated with massive intravenous infusions of one-percent NaCl. The higher dose is about double the lethal dose for dogs. No ophthalmoscopic examinations were reported.

Sayers et al. (1942),[65] (1944)[66] gave methanol mainly by inhalation and in a few experiments by skin absorption. Dosages were found to be subtoxic. No deviations from normal ophthalmoscopic findings were seen in any of the dogs.

It is of great interest that the ophthalmologist in this study, J. G. Linn, examined 30 normal control dogs with the following varied findings: no abnormalities to slight congestion of the discs; slight to marked congestion of the discs and fundi; granular eyegrounds; pallor of fundi; pallor and fuzziness of the discs; one with slight excavation of the disc; and one with an exudate. Linn believed that slight congestion which may have slightly increased after methanol was on a vascular basis due to reactions to fright or struggling.

Fink (1943)[67] concluded that he had demonstrated ganglion-cell degeneration and edema of the nervehead in histopathologic sections of dogs and rabbits poisoned with repeated doses of methanol. He found no apparent visual disturbances. Experimental details were not published, but Dr. Fink most kindly made available to us the protocols of his experiments. Five rabbits were given approximately (taking average rabbit size to be 2.0 kg.) 4.0 gm. of 100-percent methanol/kg. every other day, six doses. Slightly dilated or tortuous vessels were noted in all. Four dogs were given 3.5 gm. of 100-percent methanol/kg. every other day, five doses. On one day, in one dog, dilated retinal vessels were seen. We assume that Fink felt the appearance of the retinal vessels was not significant since he reported negative results in his published papers. Four rabbits received about 1.0 gm. of 100-percent methanol/kg. every third day, 20 doses, and four dogs received 0.66 gm. of 100-percent methanol/kg. every third day, 20 doses, without either series showing ophthalmoscopic changes. Doses in these last two groups were less than those given by authors previously reporting in vivo ocular changes after chronic poisoning.

Röe (1948)[1] found no retinal ganglion-cell changes in rats poisoned with single oral doses or in rabbits poisoned with single and repeated oral doses. As previously noted, his rat doses were low. He made no report of clinical ocular findings.

Fanta and Mayer-Obiditch (1953)[68] reported deposition of an acidophilic material into sheaths and perivascular connective tissue of the optic nerve in an unspecified number of rabbits who were killed apparently

only several hours after eating an unspecified amount of the methanol which was poured into their food. These authors stated, "There occurred again after the shortest time paralyses in the region of the rear extremities and various signs authorized the assumption that the sight of the animals was disturbed." This was their only mention of clinical ocular findings; so from the context we believe the "various signs" were early narcotic effects such as ataxia and pupillary changes, rather than those of toxic amblyopia.

Marconcini (1953)[49] claimed that subconjunctival injections of hydrogen peroxide apparently ameliorated the histopathologic changes in the optic nerves of rabbits after single systemic doses of methanol. He reported "intense hyperemia of the vessels of the ocular fundus" in three out of his series of four rabbits. One rabbit was given 3.0 gm. of 15-percent methanol/kg., intravenously; the other three were given 2.5 gm./kg., intravenously. Noè[56] reported 4.2 gm. of 20-percent methanol/kg. was the intravenous minimum lethal dose for rabbits. Thus Marconcini's doses were low.

III. Summary

A. Much of the experimental work, from which methanol-induced acidosis in nonprimates is claimed, is technically inadequate. The cases shown in Table 1 have had CO_2-combining capacity determinations made before and after methanol poisoning. This is in contrast to the frequent development of severe acidosis in humans following single oral doses. The numbers involved do not constitute adequate proof or disproof of a similar frequent development of acidosis in nonprimates.

B. Although there are many claims in the literature of clinical visual loss in experimental animals, some are erroneously based on four common sources of confusion which are not related to the typical methanol amblyopia seen in humans. Other papers are inconclusive because (1) animals were given doses which were probably subtoxic or (2) insufficient evidence was reported. Some papers reporting negative results did not include ophthalmoscopic examinations.

Five authors[22, 34, 35, 47, 51, 67] reported negative clinical ocular and ophthalmoscopic findings in rats, rabbits, dogs, and two rhesus monkeys. Almost all of these animals were chronically poisoned by oral administration. One paper[50] reported negative findings in brightness discrimination with rats.

One paper[40] reported positive clinical and ophthalmoscopic changes in chronically poisoned rabbits. We mentioned the possibility that these changes could be on the basis of confusion of appearance in view of myelinated nerve fibers, rather than on the basis of a true toxic amblyopia.

One paper[35] reported one rhesus monkey, fully documented and reasonably incontesta-

TABLE 1

Cases in the literature showing CO_2 combining capacity determinations before and after methanol poisoning

Species	Number of Animals	Dosage	Result	Acidosis	Reference No.
Dog	3	Repeated	Fatally poisoned	Severe	21
Dog	2	Single	Fatally poisoned	None	15
Dog	1	Single	Nonfatally poisoned	Moderate	15
Dog	1	Single	Nonfatally poisoned	None	21
Rabbit	3	Repeated	Exper. killed but probably fatal doses	None	1
Rabbit	2	Single	Exper. killed but probably fatal doses	None	1
Rat	9	Single	Exper. killed but probably sublethal doses	None	1

ble, with clinical and ophthalmoscopic evidence of toxic amblyopia caused by methanol.

C. Authors' summaries of histopathologic findings in experimental animals following methanol poisoning were listed. Some reported positive findings, mainly of retinal ganglion-cell and optic-nerve degeneration; others were of the opinion that these changes could all be accounted for by autolysis and fixation techniques. We did not make a critical evaluation of histopathologic experiments.

Material and Methods

I. Animals

Rats were male albinos of the Sprague-Dawley strain.

Rabbits were male albinos.

Dogs were mongrel males obtained from the city pound.

Monkeys were wild male rhesus macques (macacus malata), all apparently in good health at the beginning of experiments. Twelve days was the minimum stay in the laboratory prior to being used in experiments. During experiments they were lodged in an air-conditioned room and were given daily multivitamin supplements.

II. Solutions

A. Methanol, Merck, 99.5-percent reagent methanol, acetone free, was used throughout. Concentrations were chosen after consideration of two factors:

1. Probable stomach capacity, since a volume exceeding this would cause regurgitation.

2. Tendency of high concentrations to cause vomiting. Since toxic doses of methanol are so high in nonprimates, choosing a successful procedure was sometimes difficult. Most rodents got 50-percent methanol (weight); whereas, dogs got 16 to 26-percent, and monkeys got five to 22-percent.

B. Sucrose. Methanol solutions for dogs and monkeys contained up to 20 gm. of sucrose. The purpose in adding the sucrose was prevention of methanol irritation to the gastric mucosa, which is conducive to vomiting. Since the monkeys showed no signs of nausea or vomiting, this could well be discontinued in future monkey experiments. However, most dogs were nauseated. Apparently the sucrose, plus rapid return to feet and elevating the mouth at the end of gavage, plus flattery, kept early vomiting from occurring in all but two of the dog experiments. Keeney and Mellinkoff[70] "postulated that glucose may be a valuable adjunct to alkalinization" in the treatment of methanol poisoning, but Clark and Gibson[20] found glucose alone did not lessen toxic effects of methanol in dogs. Since food intake before experiments was unregulated in our animals, we would not expect the amounts of sucrose given to have any significant additional systemic effect.

III. Route of administration

Methanol solutions were given by gavage to unanesthetized animals, except for the first four rabbit experiments in which it was given intravenously. For rats, a curved steel needle with a bulbous tip was introduced into the esophagus; jaws were held open with string. Rubber catheters introduced orally between wooden mouth gags were used in rabbits (size 12, French) and dogs (size 26, French). Size-8 French catheters were introduced nasally in monkeys, with jaws held firmly closed.

IV. Acidosis studies

A. *Apparatus for carbon dioxide combining power determinations*

The Van Slyke manometric apparatus and technique was used for the first five rabbit experiments.

The Lazarow microgasometric apparatus[71] was used for all other CO_2-combining capacity determinations. This method[72] in our hands had the advantage over the Van Slyke

of greater reproducibility, greater rapidity, and the requirement of less blood.

In all listings of CO_2-combining capacities, all single results are averages of the two closest replicate determinations except when these had an average deviation of more than five percent. In that case the closest replicate determinations are also listed.

B. *Blood specimens*

1. Rats were killed by decapitation with sheep emasculating shears with the sharp edge toward the body. Mixed arterial and venous blood was then collected from drippings from the neck vessels.

2. Rabbits. In the early experiments in which the Van Slyke was used, blood was withdrawn after cut downs from the jugular and by heart puncture. In later experiments bloods were obtained when possible from razor cuts of the ear artery; on a few occasions they were gotten by heart puncture.

3. Dogs. Bloods came from the external jugular or the leg veins.

4. Monkeys. Bloods were obtained from leg veins when possible; otherwise from the femoral.

Any possible variations in the CO_2-combining capacities due to varying sources of blood would be expected to be insignificant beside the 20 to 30 vol. percent drop which occurs in methanol acidosis.

Bloods were collected with a drop of aqueous solution of U.S.P. Heparin (sodium salt) as anticoagulant and centrifuged on the day they were drawn. The separated plasma was kept frozen until the CO_2-combining capacity determinations were made.

Results

Our experiments dealt with acute methanol poisoning by single oral doses in rodents, dogs, and the rhesus macaque. In a few rabbits the intravenous route was used. Four types of observations, contrasting the differences between primates and nonprimates, are presented: (1) Levels of toxic doses, (2) clinical symptoms, (3) eye findings, and (4) acidosis studies. Complete data are reported in Tables 2 through 7.

I. Lethal doses

Lethal doses for rodents and dogs were six to 10 times those for humans. Monkeys had lethal doses in the same range as humans. All toxicity data now being considered are for single oral doses. Our primary interest in this study was to give lethal doses which would permit the animals to survive for 24 hours. In order to achieve this we necessarily accumulated our own toxicity data. Data from the literature are listed in Table 8.

A. *Rodents*

1. Rats. In a series of 23 rats, 9.5 gm. of 50-percent methanol per kg. was the approximate LD_{70}.

2. *Rabbits.* While we did not run a toxicity series on rabbits, we found that out of three rabbits, all died between 24 hours and three days when given 7.0 gm. of 30-percent methanol per kg. orally. One rabbit given 7.0 gm. of a 50-percent solution died in less than 24 hours.

B. *Dogs*

We had nine dog experiments, using 16-percent to 26-percent by weight methanol, with these results:

Oral Dose (gm./kg.)	Results
2.5	Survived
3.5	Survived
4.0	Died 29-46 hr.
4.5	Survived
5.5	Survived
6.4*	Survived
7.0	Survived
8.0	Survived
9.0+*	Died 28-42 hr.

C. *Monkeys*

Acute oral toxicity studies on the rhesus macaque have not previously been reported.

* Estimated because of early vomiting. See Table 6.

Our results using single oral doses of methanol were:

Oral Dose (gm./kg.)	Results
1.0	Survived
2.0	Survived
3.0	Died 32-38 hr.
4.0	Died 29-36 hr.
6.0	Died at 29 hr.
8.0	Died 6-23 hr.

This series is of course too small to give more than approximate lethal doses, especially since, as with humans, nonhuman animals in a given species probably show considerable individual variation to methanol poisoning.

II. General clinical picture

Previous reports on the clinical picture of methanol poisoning have not emphasized the difference between primates and nonprimates—a difference as great as two separate diseases. The first 24 hours after poisoning provided the sharpest contrast in that rodents and dogs getting much less methanol than the minimum lethal dose showed severe symptoms; whereas, a monkey receiving a lethal dose might have no symptoms during the first day except questionable mild intoxication.

The general clinical picture seen in various rodents and dogs was essentially the same. In addition to the animals reported in this paper, our report of clinical findings is also drawn from previous work[73] using hundreds of mice given methanol intraperitoneally.

In nonprimates, as might be expected from the higher lethal doses, narcosis was a predominant symptom. Usually in about a half hour to an hour after oral administration of methanol (less when given intravenously or intraperitoneally) intoxication occurred, as evidenced by varying degrees of ataxia and hypermotility, plus always a change in mental status toward marked happiness and amiability. Previously vicious individuals could be extensively handled without gloves.

From about an hour to several hours after administration of methanol (dependent upon the dose as well as the route of administration), the animals became semicomatose or comatose. Semicoma was considered to be present when no spontaneous motion occurred. Coma consisted of progression to absence of response to pain. Coma lasted from several hours up to four days. Eyelids were kept open and survivors of lengthy comatose states developed exposure keratitis. Most deaths occurred without recovery from semicoma or coma. In general, the picture was one of early onset of severe symptoms continuing unabated (no latent period) until death.

In contrast, rhesus macaques reacted clinically to methanol just like humans. Below a lethal dose, and occasionally with one, they did not get very intoxicated or show any other symptoms. Usually occurring with a lethal dose, monkey inebriation showed almost every type of individual variation which can be seen in intoxicated humans, although increased amiability was not obvious.

Semicoma appeared on the first day only in the monkey getting over two and one-half times the minimum lethal dose and dying probably around 12 hours after methanol. The morning following the administration of methanol usually found the monkeys apparently normal. This was identical with the latent period in humans.

Later on in the second day, monkey pull on chain, which had been slightly diminished during inebriation but had recovered the morning after, would again weaken. The resistance exerted by a monkey to a pull on his chain was a surprisingly good index of his physical well-being. Pull on chain progressively weakened in fatal cases. Eventually, the monkey became sick enough to lie down during daylight in the presence of humans. Semicoma was seen only shortly prior to death. Deaths occurred from respiratory failure.

According to the literature, humans react

to methanol in the same way as monkeys.

III. Eye examinations

Pupillary changes and exposure keratitis were mentioned in our review of the literature. With the onset of narcosis in nonprimates, occurring an hour or so after poisoning, pupillary changes sometimes occurred: mydriasis or miosis, with or without sluggish and sometimes absent reactions to light. During coma eyelids were almost always open; and, when coma lasted about a day or longer, exposure keratitis, secondary infection, and corneal opacification frequently occurred. Two monkeys showed dilated, unreacting pupils a few hours prior to death. They had slight ophthalmoscopic changes, but they were also semicomatose. Consequently, there can be no certainty as to whether pupillary changes in these monkeys were due to toxic amblyopia or to a narcotic effect.

Positional horizontal nystagmus was observed in nonprimates. This was unfortunately not studied in our monkeys; however the literature contains reports of positional nystagmus, similar in character to that seen in our nonprimates, observed in humans poisoned by methanol and ethanol (Menne,[74] Gorman,[75] Meyer zum Gottesberge[76]).

Some authors briefly mentioned the observation of nystagmus in nonprimates after poisoning with methanol and with higher alcohols.[29, 30, 37, 41, 52, 53] The nystagmus developed usually about a half hour after administration of methanol during the intoxicated stage. It was usually present only with the animal's head in the lateral position. When occasionally present in other positions, it was more severe in the lateral position. At first the quick component of the horizontal nystagmus was always down with the animal's head in the lateral position. Duration was from several hours to two days. When it persisted for a day, after the first day in a few instances the quick component of the horizontal nystagmus was up when the animal's head was in the lateral position. No monkeys showed nystagmus in the erect position, but none were examined early in the lateral position.

Repeated ophthalmoscopic examinations were made only on monkeys, dogs, and the rabbits receiving methanol by gavage. One moribund rat was examined; but the observer believed that, although fundi seemed normal, magnification was insufficient for certainty.

Myelinization of retinal fibers in the rabbit makes difficult the appraisal of minor changes involving the discs. However, under our experimental conditions, no rabbits showed any fundus changes.

None of our dogs showed any ophthalmoscopic changes. The disc margins of many dogs normally appeared fuzzy because of tiny irregularities in bordering pigmentation.

Two out of six monkeys, both receiving lethal doses, showed eyeground changes. One developed a small monocular retinal hemorrhage one-half disc diameter temporal to the disc just prior to death and 29 hours after administration of 6.0 gm. methanol/kg. The other, given 3.0 gm./kg., showed at 25½ hours, slight but definite blurring of the temporal disc margins and questionable retinal venous engorgement, O.U. At 31½ hours disc margins were blurred everywhere except nasally, there was possible hyperemia of the discs, and veins near the disc had a diameter estimated to be triple that of the accompanying arteries. Death occurred between 31¾ and 37½ hours, with no examinations made after 31½ hours. At the time eyeground changes were seen, both of these monkeys were too weak to resist handling; thus, there was no question of vascular changes induced by neck stricture.

IV. Acidosis studies

A. *Rats*

Nine white male rats of similar ages were given 9.0 gm. of 50-percent methanol/kg.—approximately the rat oral LD_{10}. Bloods were obtained, each time in three animals, at 4½, 27, and 47 hours after administration

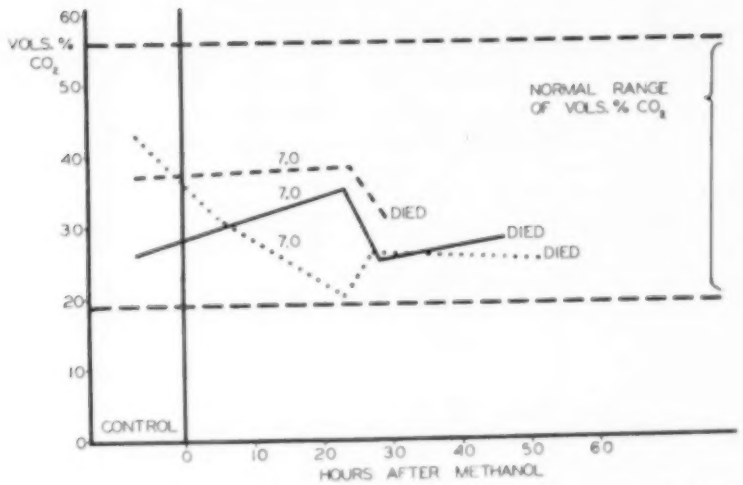

Fig. 1 (Gilger and Potts). Time course of plasma CO_2-combining acapacity in rabbits after 7.0 gm./kg. oral methanol.

of methanol. Their CO_2-combining capacities ranged from 47 to 80 vol. percent.

B. *Rabbits*

Rabbits were found to be very poor subjects for acidosis studies. There was a tremendous variation in normal CO_2-combining capacity, not only between different animals, but also in the same rabbit over the course of control periods lasting up to three weeks. Our rabbits had a normal variation from 19 to 56 vol. percent.* Our five experiments using anesthetized rabbits and drawing repeated 5.0 to 10 cc. blood samples are reported only in the tables. In addition, these experiments lasted less than eight hours which probably did not give sufficient time for the development of acidosis. Suffice it to say here that none showed severe acidosis following methanol.

In four rabbit experiments, 7.0 gm. methanol/kg. was given by gavage to unanesthetized animals, and 1.0 to 2.0 cc. blood samples were drawn. One of these rabbits, receiving a higher concentration than the others, died in less than 20 hours which might not have allowed him sufficient time to develop acidosis.

The CO_2-combining capacity determinations obtained on the three remaining rabbits are shown in Figure 1. The lines begin in the control period at the mean normal CO_2-combining capacity for each animal. Methanol produced no values less than the normal rabbit range. The dotted line rabbit showed a greatest postmethanol drop from his last normal value immediately preceding administration of methanol of 16 vol. percent; whereas, he showed a normal variability of 20 vol. percent.

C. *Dogs*

Figure 2 shows our experience with dogs. It is the same type chart as the preceding one, except that our dogs showed a normal range of only 12 vol. percent among differ-

* Fröhlich[7] found a normal range in rabbits from 26 to 58 vol. percent; he also reported blood loss of 6.0 cc., three times inside of 24 hours caused decrease in rabbit CO_2-combining capacity. Pitt[8] reported that ether caused drops in the plasma CO_2-combining capacity of rabbits. In view of our finding of considerable individual variability in CO_2-combining capacity obtained from 1.0 cc. samples from unanesthetized rabbits inside of an eight-hour period, the conclusions of these authors may be open to question.

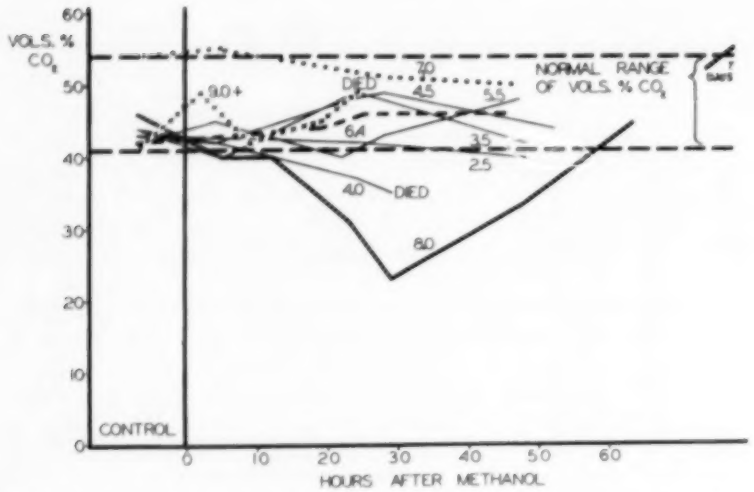

Fig. 2 (Gilger and Potts). Time course of plasma CO_2-combining capacity in dogs after oral methanol. Methanol dose (gm./kg.) indicated adjacent to each curve.

ent animals, and the variation of an individual dog over a period of weeks was insignificant. Control values, unlike those in the rabbit chart, are each from single blood samples drawn shortly before the administration of methanol. Our dogs receiving doses lower than 6.3 gm./kg. are charted with narrow unbroken lines.* All of these dogs showed toxic symptoms, but their lack of acidosis should not be given as much weight as in the others. The slight drop shown by the 4.0-gm. dog who died is insignificant when compared with monkeys or people. The dogs represented by heavier lines all received doses which, according to the literature, should have killed them. Only the dog (line of dashes) who received 8.0 gm./kg. and survived, developed acidosis. His lowest CO_2-combining capacity was 23 vol. percent. The thick solid-line dog, who received over 9.0 gm./kg. and died after 24 hours, maintained a normal acid-base balance.

The possibility occurred to us that dogs as a species might be resistant to the development of acidosis. Therefore, one of our surviving dogs was given dilute HCl orally. Inside of three hours his CO_2-combining capacity had dropped over 20 vol. percent.

D. *Monkeys*

Monkeys again reacted like humans (fig. 3). Our first monkey receiving 8.0 gm./kg. is not charted because we got only one blood sample due to our inexperience with the clinical course of methanol poisoning in primates at that time. Survival occurred only with 1.0 and 2.0 gm./kg. With 1.0 gm.—the dot-dash line—the alkali reserve was unchanged. With 2.0 gm—the solid line—the CO_2-combining capacity dropped by 24 hours to 16 vol. percent and by 48 hours had begun to rise. This 2.0-gm. monkey made quite a contrast with our 7.0 gm./kg. dog.

The dog, after getting horribly drunk, was comatose for 24 hours. He was unable to stand for three days. His CO_2-combining capacities varied during this time between 50 and 54 vol. percent.

The 2.0 gm./kg. monkey was not intoxi-

* In the only sizeable series[10] of dogs given single oral doses of methanol, 6.3 gm. of 100-percent methanol/kg. was found to be the LD_{50}.

cated. The only clinical finding was questionable impairment in grasp.

The monkeys getting lethal doses of 3.0, 4.0, and 6.0 gm./kg. had CO_2-combining capacities decreased by 24 hours to less than 15 vol. percent; and death followed in all three cases without recovery from the severe acidosis.

Conclusions

I. Lethal doses

We found the approximate single oral lethal doses to be:

Animal	Times mean human lethal dose
1. Rats	9
2. Rabbits	7
3. Dogs	9
4. Monkeys	3

These doses in rats, rabbits, and dogs are comparable to the figures available in the literature.

Harnack[19] in 1912 was impressed by the fact that with single lethal doses methanol was the least toxic of the aliphatic alcohols in experimental animals, whereas in man so little methanol could cause blindness and/or death. From this he concluded that methanol reacted differently in different animals. We are in entire agreement with Harnack but would differentiate mainly between primates and nonprimates. Nonprimates required seven or more times the average human lethal dose. Death occurred in our monkeys at 3.0 gm./kg.; whereas humans have been reported as having survived 2.9 gm./kg.[27] Our series of monkey experiments was too small to get accurate toxicity figures but obviously monkey single oral lethal doses are of the same order of magnitude as those for humans.

II. General clinical picture

The general clinical picture of methanol poisoning in primates and nonprimates was that of two different diseases. Nonprimates showed severe early intoxication and narcosis; narcosis lasted until death. Primates showed much less intoxication than nonprimates and much less than primates affected by ethyl alcohol. They then had a symptomless latent period followed by sickness and death. Narcosis appeared only as a terminal manifestation.

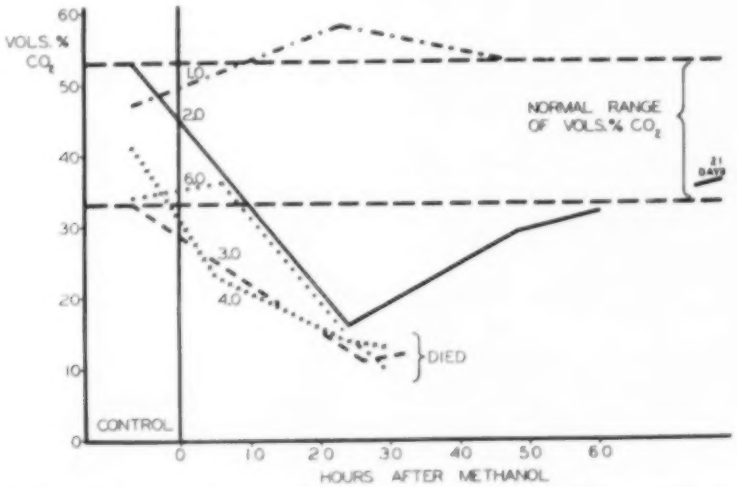

Fig. 3 (Gilger and Potts). Time course of plasma CO_2-combining capacity in monkeys after oral methanol. Methanol dose (gm./kg.) indicated adjacent to each curve.

III. Eye examinations

Early pupillary changes and corneal opacification following exposure keratitis in nonprimates are considered to be due to the central narcotic effects of methanol and not to toxic amblyopia.

The positional horizontal nystagmus produced by methanol has only been of passing interest to most investigators, many undoubtedly overlooking it. Our observations have been inadequate to illuminate the mechanism whereby this is caused.

Eyeground changes were not seen in nonprimates following methanol poisoning but were seen in primates.

IV. Acidosis studies

Our rats showed no acidosis from methanol. Wide normal variability in rabbit CO_2-combining capacities makes them unsuitable for acidosis studies. Under our experimental conditions, methanol did not cause acidosis in rabbits. Only one dog out of nine experiments developed acidosis. The rhesus macaque usually developed severe acidosis.

Thus again we found a contrast in that in nonprimates acidosis occurred rarely whereas in primates severe acidosis developed frequently.

Summary

Both the literature and our experiments indicate that only in primates is there close similarity in response to methanol poisoning with respect to: (1) Levels of toxic doses, (2) generalized clinical symptoms, (3) clinical ocular pathology, and (4) frequent development of acidosis.

University Hospitals (6).

We wish to acknowledge the assistance of Mrs. C. Stuart in locating many of the papers discussed and of Mr. S. Rehmar for translation from the Russian of reference 40 and of Mr. K. Kurahashi for translation from the Japanese of reference 63.

TABLE 2

I. Rats: A. Acute oral toxicity experiments

Experiment No.	Rat Weight (gm.)	Dosage (gm. methanol/kg. rat)	Results
23	478	3.0	Survived
	86	3.0	Survived
	523	5.5	Survived
	87	5.5	Survived
	538	8.0	Survived
	70	8.0	Survived
29	386	9.0	Survived
	347	9.0	Survived
	202	9.0	Survived
27	362	9.5	Survived
	324	9.5	Died 22–23 hr.
	388	9.5	Died at 6 da.
	346	9.5	Died 22–25 hr.
	316	9.5	Survived
	370	9.5	Died 49–68 hr.
26	391	11.0	Died 23–25 hr.
	305	11.0	Died 3½ da.
	411	11.0	Died 43–47 hr.
	391	11.0	Died 2½ da.
	393	11.0	Died 25–43 hr.
	375	11.0	Died 47–49 hr.
25	550	11.0	Died 29 hr.
	523	14.0	Died 3 hr.

Methanol was given in a 50-percent (by weight) solution.

At 3.0 gm./kg. no clinical symptoms. From 5.5–9.0 gm./kg. increasing severity of ataxia, no coma. At 9.5 gm./kg. all showed severe ataxia and two were comatose while under observation. At 11.0 gm./kg. all had severe ataxia; 6 out of 7 were comatose. At 14.0 gm./kg. the rat showed severe ataxia and coma. Nystagmus was present in many.

TABLE 3

I. Rats: B. Acidosis study

Rat No.	Rat Weight (gm.)	Decapitated at (hr. after methanol)	Plasma CO_2 Combining Capacity (vol. %)
1	291	4½	64.4
2	265	4½	46.8
3	237	4½	60.8
4	291	27	80.4
5	286	27	59.1
6	182	27	64.8
7	323	47	77.6
8	294	47	54.7
9	230	47	55.4

Each rat was given a single oral dose of 50 percent methanol/kg. body weight. All were severely ataxic and had nystagmus; two were semicomatose.

TABLE 4

II. Rabbits: A. Plasma CO_2-combining capacity of rabbits under anesthesia with and without methanol

(Methanol was given in 50-per cent solution intravenously. The Van Slyke manometric apparatus was used for CO_2 determinations.)

Experiment No.	Rabbit Weight (kg.)	Anesthetic Given	Time after Start of Experiment (min.)	MeOH (gm./kg.)	Total MeOH (gm./kg.)	CO_2 capacity (vol. %)	Remarks
3		Nembutal	10	0		50.3*	Died 65 min. after start of experiment
			20	0		51.2	
			50	0	0	25.7 (27.2 / 24.2)	
4	3.5	None	0	4.2			Died 25 min. after start of experiment
			20	0		47.4	
			25	0	4.2	45.1	
5	3.4	Nembutal	25	0		52.2	Apnea for 5 min. after MeOH; recovery with artificial respiration. Died 40 min. after start of experiment
			30	1.5			
			40	0.7	2.2	31.7	
6	3.6	Drop ether	35	0		23.2	Died 150 min. after start of experiment
			40	0.7			
			50	0.7			
			70	0		36.1	
			80	2.6			
			140	0		46.6	
			150	1.4	5.4	53.9	
7	3.5	Drop ether	45	0		19.0	Killed 380 min. after start of experiment
			70	4.0			
			140	0		17.9 (19.0 / 16.8)	
			195	1.0			
			210	1.0			
			225	1.0			
			240	1.0			
			255	1.0			
			270	1.0			
			285	1.6		34.7*	
			345	0		21.8*	
			380	0	11.6	21.8*	

* Insufficient plasma for duplicate determinations.

TABLE 5

II. Rabbits: B. Experiments giving methanol by gavage without anesthesia and using Lazarow apparatus

Experiment No.	Weight (kg.)	Dosage MeOH (gm./kg.)	Time Normal Bloods Drawn (days)	Time Drawn after MeOH (hr.)	CO_2 capacity (Vol. %)	Eye-grounds	Died (hr. after MeOH)	Remarks
41	2.7		1		25.8	Normal		Ataxia. Comatose from 2–22 hr.; semi-comatose thereafter until death. Nystagmus. At 3 hr. pupils unreactive; from 23 hr. on pupils reactive. Died while sample 4 was being drawn. Immediate heart puncture. 46 hr. point on Figure 1 is average of samples 4 and 5.
		7.0 (40%)		23	35.0	Normal		
				28	25.2	Normal		
				46	26.4 (28.0 / 24.8	Normal	46	
				46	30.5			
42 & 44	1.9		1		39.0	Normal OS		Ataxia. Comatose from 1–5 hr.; semi-comatose thereafter until death. Nystagmus. Had previous surgery, O.D.
			2		40.3			
			15		56.2			
			21		36.5			
		7.0 (40%)		5	31.4	Normal		
				23	20.4	Normal		
				27	25.7	Normal	Between	
				51	24.6	Normal	52 and 69	
45	2.8		1 (a.m.)		41.4	Normal		Ataxia; semicomatose from 4 hr. until death. Nystagmus.
			1 (p.m.)		28.6			
			6		41.1			
			13		39.9			
			20		35.2	Normal		
		7.0 (40%)		24	37.5		Between	Pupils moderately dilated.
				29	30.8	Normal	29 and 48	

TABLE 6

III. CO_2-COMBINING CAPACITY OF DOGS GIVEN SINGLE ORAL DOSES OF METHANOL

Experiment No.	Weight (kg.)	Dosage MeOH (gm./kg.)	Percent MeOH (by weight)	Time after MeOH (hr.)	CO_2-capacity (vol. %)	Eyegrounds	Remarks
22	19.9	2.5	17	0	43.2	Normal	Slight ataxia from 1-5 hr. Nystagmus. Somnolence. Recovery by 5½ hr.
				24	41.7	Normal	
				48	40.0		
24	16.7	3.5	21	0	44.3	Normal except disc O.D. pink disc O.S. gray	Severe ataxia ½–4½ hr. Somnolence. Recovery by 24 hr.
				5	40.3		
				24	48.2	No change, O.U.	
				48	42.1	No change, O.U.	
16	8.2	4.0	16	0	43.7	Normal	Marked ataxia after 15 min. Somnolence. Nystagmus. By 24 hr. severe hangover (weakness, ataxia, somnolence, "dry heaves," the "shakes"). Because of coughing was examined by veterinarian who reported normal temperature and lungs clear to percussion and auscultation. Died between 29 and 46 hr. No autopsy.
				5	41.6	Normal	
				24	37.2	Normal	
				29	35.3		
28	17.3	4.5	24	0	42.5	No change	Used 30 days previously in Experiment 24. Marked ataxia ½–23 hr. Marked euphoria. Somnolence. Recovery by 28 hr.
				4	42.4		
				23	48.3		
				28	49.4		
				52	43.9	No change	
31	13.5	5.5	25	0	42.4	Normal	Euphoria and ataxia at ½ hr. Comatose with opisthotonos 3–22 hr. Nystagmus. At 22 hr. could not walk. At 28 hr. could walk but not navigate stairs. Recovery by 47 hr.
				4	44.5		
				22	40.2		
				28	42.9	Normal	
				47	47.8		
32	13.5	Given (O) 200 cc. of 6% (by vol.) HCl.		After HCl 0	47.0		Dog used 34 days previously in Exper. 31. About 100 cc. vomited in first half hr. About 30 cc. bright red vomitus at 2½ hr. Diarrheal stools from 4–6 hr. Recovery by 24 hr.
				3	26.7		
				6	30.5		
34	13.4	6.4	24	0	41.8	Normal	Dog used 7 days previously in Exper. 32. Given 6.5 gm. MeOH/kg. Immediate regurgitation of an estimated 30 cc. Dosage thus 6.4 gm./kg. Ataxia and euphoria beginning at 45 min. Unable to stand. Nystagmus. Hangover at 24 hr. Recovery by 44 hr.
				19	43.5		
				25	46.3	Normal	
				45	45.8		
46	11.2	7.0	26	0	54.1	Normal	½ hr.—ataxia and euphoria
				5	54.8		1 hr.—semicomatose
				23	51.7	Normal	3–25 hr.—comatose
				29	51.4	Normal	25–27 hr.—semicomatose—pupils 1–2 mm. diameter. 27–46 hr.—comatose
				46	49.5	Normal	
				3 da.	49.3	Normal	3–5 hr.—convulsive running movements of legs. Positional nystagmus early.
				8 da.	53.7	Normal	96 hr.—able to stand. Recovery by 7 da.
47	12.4	8.0	27	0	46.2	Normal	10 min.—ataxia and hyperactivity
				5	39.9	Normal	30 min.—unable to stand. Nystagmus. 1½ hr.—4th day—comatose, lids open, exposure keratitis. 1½–2½ hr.—conjugate deviation of eyes to left; running leg movements. 4–5 hr.—pupillary reactions slight or absent; 24 hr.—quick component of nystagmus reversed in direction; peripheral shock. 47 hr.—nystagmus continued. 7th day—tried to sit; too weak to walk. 15th day—could walk; left foreleg 3× size of right. Both corneas opaque obscuring fundi.
				12	40.3		
				28	50.7	Normal	
				29	25.3	Normal	
				47	32.9	Normal	
				7 da.	55.4	Normal O.D. Cornea O.S. opaque	
49	11.4	9.0+	26	0	41.1	Normal	1 hr.—had vomited a vol. estimated to be 100 cc. greater than gavage. Moderate ataxia. Re-gavaged with identical solution (9.0 gm./kg.) at 70 min. No vomiting after 2nd gavage =9.0+gm./kg. 10–15 min. after 2nd gavage—comatose; nystagmus. 19 hr.—still comatose—exposure keratitis—2 mm. pupils. Death occurred between 28 and 42 hr. without recovery from coma.
				2	48.8	Normal	
				9	42.2		
				19	44.6	Normal posterior pole	
				24	48.6	Normal	

TABLE 7

IV. CO_2-COMBINING CAPACITY OF MONKEYS GIVEN SINGLE ORAL DOSES OF METHANOL

Experiment No.	Weight (kg.)	Dosage MeOH (gm./kg.)	Time after MeOH (hr.)	CO_2-capacity (vol. %)	Eyegrounds	Remarks
19	2.72	1	0	46.7	Normal throughout	No general clinical symptoms
			21	58.1		
			48	53.3		
21	2.19	2	0	52.7 (ave.)	Normal throughout	Monkey used 15 days previously in Exper. 19. No detected clinical symptoms with the following possible exception: At 24 hr. he got partly loose and failed to bite the observer. Subsequently became a pet, showing no evidence of impairment of vision. Died 6 mo. later from acute miliary tuberculosis
			24	15.9		
			48	28.7		
			21 da.	36.3		
20	3.45	3	0	32.6	Normal	No definite signs of intoxication. At 25½ hr. sick, weak, had to lie down after struggling. Pupils dilated but reacted to light. At 33½ hr. sicker; lying down; made only rare spontaneous movements; pupils dilated and unreactive; hippus present; did not blink at threatening gestures but was semicomatose. Died 34–39½ hr. Rigor mortis present at 39½ hr.
			8	25.3	Normal	
			26	10.6	Blurring temporal disc margins, O.U. Questionable venous engorgement, O.U.	
			34	12.4	O.U.:—disc margins blurred except nasally. Retinal veins had diameter ⅔ × that of arteries; possible hyperemia of discs	
17	2.88	4	0	41.5	Normal throughout	Ataxia marked by 75 min. At first quiet but by 3 hr. was combative. Apparently mentally alert, although pull on chain weaker. At 22½ hr. rested head against side of cage. At 29 hr. lying down; conscious. Death occurred between 29 and 36 hr.
			5	23.0		
			23	13.9		
			29	12.7		
16	3.32	6	0	33.7	Normal	Ataxia marked by 80 min.; pull on chain weak
			6	35.6	Normal	At 23 hr. stood and was apparently normal
			24	14.4	Normal	At 24 hr. lying down and very weak
			28	11.3 (10.2, 12.1)	Normal	At 27 hr. became semicomatose, remaining so until death
			29	9.8 (10.8, 8.7)	Pinpoint retinal hemorrhage temporal to disc in one eye	At 29 hr. pupils were dilated and unreactive
			29½	14.4		Death at 29½ hr. following respiratory failure
15	2.76	8	5	39.2	Normal throughout	Ataxia marked by 70 min.; increasingly weak
						At 2 hr. lying down; very sick
						At 3½ hr. semicomatose, remaining so
						Death occurred between 6 and 23 hr.; at 23 hr. rigor mortis was pronounced

TABLE 8

ORAL SINGLE LETHAL DOSES OF METHANOL IN NONPRIMATES

Species	Dose gm./kg.	% Methanol	Result	Number of Animals	Source
Rat	8.3	70	LD_{50}	48	Alder, et al.[33]
Rabbit	7.2	?	MLD		Baer[30]
Rabbit	14.2	?	LD_{100} in less than 24 hr.		Munch and Schwartze[32]
Dog	6.3	100	LD_{50}	15	Haskell, et al.[14]
Dog	6.3	50	Approx. MLD	4	A. Leo[34]

Human lethal doses are often difficult to compute accurately. In the literature[31] there are reports that death has resulted from as little as 0.34 gm./kg. and survival has occurred after as much as 2.9 gm./kg. The dose generally accepted as lethal is about 0.85 to 1.4 gm./kg.

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THE VISUAL TOXICITY OF METHANOL*

VI. The clinical aspects of experimental methanol poisoning treated with base

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There have been a number of descriptions of the clinical picture of methanol poisoning in humans.[1-3] Due to the fact that human methanol poisoning represents a medical emergency, descriptions of any one case are necessarily fragmentary. However, the characteristics as indicated by a composite of the published reports are as follows:

After the ingestion of a fatal dose of methanol the patient becomes inebriated, though by no means as thoroughly as with a corresponding amount of ethanol. Following inebriation in all but extreme doses, there comes a latent period of six to 24 hours during which the patient may have a hangover but is not otherwise ill. This period is ended by the onset of dyspnea, asthenia, failing vision, convulsions (in some cases), coma, and death. The characteristic laboratory finding in untreated cases during this period is an increasingly severe metabolic acidosis.[3] An unidentified organic acid has been found in the urine of one case of methanol poisoning and has been shown not to be formic, lactic, or acetoacetic acid.[4]

A number of writers have emphasized the significance of the acidosis in methanol poisoning[2,3,5] and have implied that both blindness and death result from it. They claim that adequate treatment of the acidosis with base saves both life and vision. Although available statistics solidly support the life-saving role of alkali therapy, there is much question about the effect of acidosis on vision and the ability of base to mitigate the eye effects of methanol.[3]

In a previous paper of this series[6] we have demonstrated how lower experimental ani-

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mals do not show typical methanol poisoning as seen in man, but that the rhesus monkey, a primate, does reproduce the human symptoms exactly. It is the purpose of the present report to describe the clinical picture in monkeys treated with base, and to evaluate the virtue of bicarbonate therapy in regard to its effect on survival and on eye signs.

Experimental

Methods

Young adult male macaccus rhesus monkeys were given pure synthetic methanol in 30-percent aqueous solution as described previously.[6] Doses were fixed at 6.0 gm./kg. (twice the certain lethal dose) unless otherwise specified. At zero time and at suitable intervals blood was drawn from a leg vein and plasma CO_2 combining capacity was determined by the Lazarow micromanometric method.[7] In addition, when volume allowed, determinations of methanol were done by the chromotropic acid method on a dilution of a trichloracetic blood filtrate. All urine was collected in 24-hour specimens beginning 24 hours before administration of methanol. Urines were titrated for organic acid by the method of Palmer and Van Slyke using a glass electrode pH determination as the end-point rather than change in color of an indicator. Methanol content and formate determinations were done on urines from a number of animals.

At frequent intervals throughout the first three days, and at longer intervals subsequently, the animals were examined ophthalmoscopically and observed for general clinical signs.

When CO_2 combining capacity fell below 25 volume percent, sodium bicarbonate in four-percent solution was given by stomach tube. During a few later experiments it was attempted to anticipate acidosis, and at 20 hours a dose of 4.0 to 5.0 gm. of bicarbonate was given. This was supplemented only when CO_2 capacity began to fall.

Results

Results are presented in the clinical protocols (table 1). Salient features of the first four animals are shown in Figure 1. Greater detail in the case of one animal is shown in Figure 2.

Discussion

In every untreated animal investigated so far, a dose of 3.0 gm./kg. of methanol or more has been enough to cause death. When, however, the acidosis is combatted with base, the end result has been highly variable both in regard to survival and eye symptoms. The following is an outline summary of what one may expect clinically in a poisoned monkey:

Beginning about one hour after administration of 6.0 gm./kg. the monkey becomes intoxicated. His usual aggressiveness is diminished and he bends all effort to preserve his dignity and equilibrium. By four hours, intoxication is maximum, and a pull on the chain is enough to cause the animal

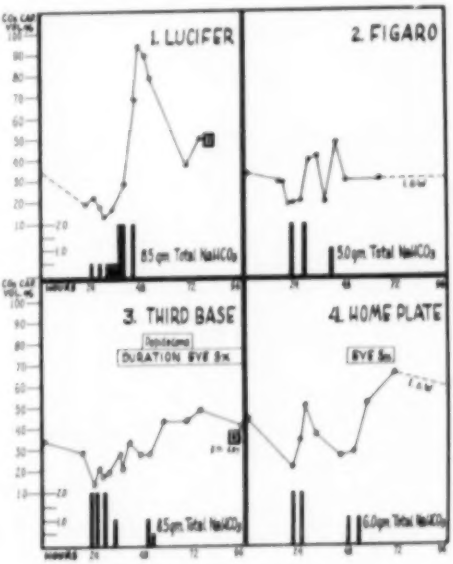

Fig. 1 (Potts). Blood base (CO_2 capacity) and base given methanol-poisoned monkeys (6.0 gm./kg. MEOH).

TABLE 1

CLINICAL ASPECTS OF EXPERIMENTAL METHANOL POISONING TREATED WITH BASE

Hr.	CO_2 Capacity (vol. %)	Base Given (gm.)	Condition of Animal
MONKEY 1, WEIGHT 4.4 kg.			
3			Very drunk; stands and pulls on chain but very weak
20			
22	19.3	0.5	
25	21.9		Seems normal
28	17.3	0.5	
30	13.5	0.5	Weak again
33 to 37	16.8	5.0	Comatose
39 to 41	28.6	2.0	Semicomatose
44	68.5		
50	89.4		Sits up weakly
70	37.6		Semicomatose
78	50.4		Dead
MONKEY 2, WEIGHT 4.2 kg.			
0	33.2		Normal
18	28.2		Hangover
21 to 22	19.4	2.0	Normal but weaker
26	21.3		Respirations 40
28		2.0	Active and only slightly weak
34	40.1		
38 to 41	20.7	1.0	No change
48	30.4		Normal
MONKEY 3, WEIGHT 4.0 kg.			
0	34.2		Normal
21	29.4		Noticeably weak
24	13.4	2.0	More energetic
29	17.5	2.0	Still energetic
32	19.2		Alert but weak, pupils dilated, contract stepwise ? Vision.
35		1.0	Peripapillary edema
37 to 45	27.7 to 32.8		Progressively weaker, wheezing
47	27.2		Extensive retinal edema blurring of disc margins
50		1.0	
53	27.9	0.5	Pupils wide
58	42.6		Animal stronger, marked papilledema, pupils wide
76	49.6		More strength, pupillary reflex returning, periphery of fundus no longer edematous—only peripapillary region still shows edema
4 days	41.0		Edema gone O.S. except for temporal disc margin; still peripapillary OD
5 days	46.9		Tremor persistent; pupil reacts to light but will not hold
6 days			No change
8 days			Cannot move
9 days			Found dead
MONKEY 4, WEIGHT 3.8 kg.			
0	43.7		Well
20	22.1	2.0	Living and well
25	34.1	2.0	No change
33	42.6		Half strength but otherwise normal; fundi normal
45 to 48	27.8	1.0	Circumpapillary edema
51 to 53	29.5	1.0	
57	52.3		Disc margin now blurred, disc normal, pupil O.D. wide and reacts poorly to light
3 days	66.7		Living and well

TABLE 1—*(continued)*

Hr.	CO_2 Capacity (vol. %)	Base Given (gm.)	Condition of Animal
MONKEY 5, WEIGHT, 3.95 kg.			
0	52.7		Well
20			Found dead
MONKEY 6*, WEIGHT 3.0 kg.			
0			Normal
21	22.2	4.0	Normally lively
24	33.4		No eye symptoms
28	32.8		
36	23.7	1.0	
38			Congestion of vessels on disc
69	33.8		Living and well
MONKEY 7, WEIGHT 5.75 kg.			
0	29.2		Normal
20	26.4	6.4	Crouched in corner
32	28.0	1.0	Perimacular edema; no extension to disc
56	27.0		Edema practically gone; animal well
MONKEY 8, WEIGHT 4.8 kg.			
0	24.0		Normal
20	27.3	4.0 (ca)	Poor pupillary reaction; hyperemia of discs
25	22.8		Pupils do not respond to light
26		1.0	
32	28.0		Retinal edema
32½			Dead

* Nine additional doses of 6.0 gm./kg. at weekly intervals with base resulted in no disc pallor or loss of vision.

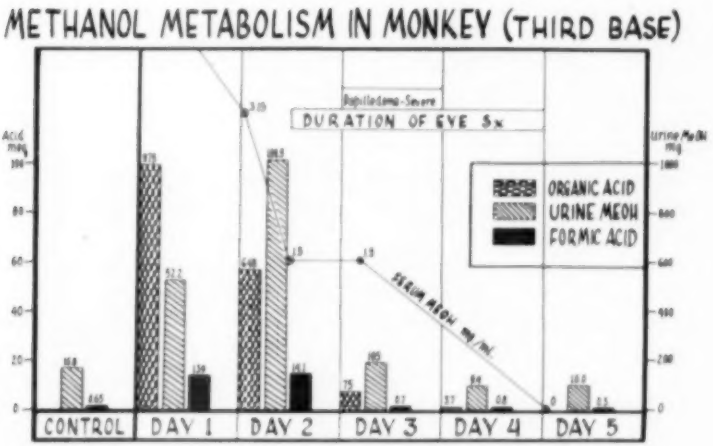

Fig. 2 (Potts). Methanol metabolism in Monkey 3 (third base).

to fall flat on his face. This phase gradually recedes, and by 15 hours the animal, though not lively, sits and eats normally and actively resists efforts to remove him from the cage. During this whole period, eye signs are normal and blood base has not changed significantly.

Between 20 and 24 hours, the blood base drops precipitously and the untreated animal becomes comatose and soon dies. If, at the first drop in CO_2 capacity, an adequate amount of bicarbonate is given and the blood CO_2 capacity is kept normal by supplements if necessary, then two alternate courses may be followed both clinically and in regard to eye signs. These events appear to be fortuitious, and eye findings seem to be independent of the rest of the clinical course.

Clinically the animal may respond well to the first base administered, continue to regain strength, and with or without additional supplements of bicarbonate become entirely well during the second 24 hours (Animals 2 and 4).

Alternatively, whether small or large amounts of base are required, the animal may show progressive weakness, and despite normal CO_2 capacity this lethargy may progress to coma and death (Animals 3 and 8).

Eye signs, too, are apparently independent of base required and certainly of degree of acidosis. Most extreme eye findings appeared in Animal 3. At about the 20th hour, peripheral retinal edema was observed in the papillomacular area. During the next 12 hours, this edema spread both toward the ora and toward the disc, and at 48 hours the entire fundus was edematous.

The fovea was clearly seen as the rhesus equivalent of the "cherry red spot." The color was closer to a chocolate brown which stood out clearly in the glistening white retina. At this time, too, the disc margin was perceptibly blurred.

By 58 hours the whole disc was elevated and edematous—the classical picture of severe papilledema. The edema receded in just the reverse order, and by 72 hours the disc appeared normal. The edema of the fundus persisted in diminishing degree for another 48 hours but, when the animal died on the ninth day, the fundi were essentially normal.

In other animals various lesser degrees of the above findings were seen from transient retinal edema after 30 hours to more moderate papilledema. Transient edema was seen in Monkeys 1 and 2, but was not recorded because the phenomenon did not persist. There is no correlation between amount of acidosis and eye symptoms.

Early in the period of retinal edema in the more susceptible animals, the pupil shows a stepwise contraction. Instead of contracting smoothly and rapidly the pupil contracts perhaps one fourth of the total extent, then partly dilates, then reaches one-half size, then partly dilates again. Thus, in four or more steps, the pupil reaches full contraction. When the light stimulus is removed, the same process occurs in reverse. It is hard to postulate a neurologic mechanism for such happening, but the phenomenon has been observed enough times to establish it as real. A similar phenomenon has been observed in hysterics by Lowenstein and Friedman.[8] Of course at a later stage, in severely affected animals, the pupil is wide and immobile.

A second phenomenon is the histopathologic finding of patchy demyelinization in the optic nerve of an animal nine days after the usual dose of methanol combatted with base. This is based on examination of one eye, and must await further studies for confirmation.

Figure 2 shows the typical picture of blood methanol content, urine methanol excretion and urinary acid production in Monkey 3. This is the animal with the most dramatic eye involvement and is used for purposes of illustration, but aside from severity of eye symptoms the results are essentially the same in all animals.

Figure 2 shows that by the time of onset of eye symptoms, and particularly of papil-

ledema, the blood methanol is negligible and most of the urinary excretion of both methanol and organic acid is over. Only a small portion of the organic acid is seen to be formic acid. Thus, even the neutralized acid is gone before eye symptoms become significant, and one finds it difficult to attribute such an effect to either methanol itself or the presence of neutralized acid.

Acidosis cannot play a part, because in animals where base is given at 20 hours and acidosis is anticipated, there is absolutely no fall in CO_2-combining capacity, but fundus edema is nevertheless observed. Further, if the degree of acidosis observed were at all significant in itself, every diabetic who goes into coma should show fundus edema and visual symptoms. This has not been observed to our knowledge.

What alternative hypothesis is available, then, to explain the effects of methyl alcohol on primates? A likely general explanation in the light of our present knowledge is the following:

Primates possess (a) a unique enzyme system capable of oxidizing methanol to some toxic intermediate or (b) possess enzyme systems particularly susceptible to the toxic intermediate produced by all species.

The metabolic defect produced by one of these mechanisms has multiple manifestations. One of these is a metabolic acidosis due to the as yet unknown organic acid. A second is the retinal edema (secondary to retinal damage) which is almost certainly not due to the acid but is parallel with its production. A third is the central nervous-system depression which in the more affected animals leads to death. The wide variation in susceptibility among both monkeys and men can be explained on variation in either process (a) or (b).

The nature of the characteristic latent period now has become obvious. It is the interval between the end of inebriation and the onset of the secondary metabolic effects. Where acidosis is untreated, the onset is most dramatic, and with doses of the size used here death occurs rapidly. On treatment with base latency is longer—until the onset of retinal edema at about 36 hours. The majority of these experiments do not have a fatal termination. In those that do terminate fatally, death is rarely sooner than three days.

Conclusions

1. In monkeys given twice the certain lethal dose of methanol, life can be saved in most cases by adequate doses of sodium bicarbonate.

2. Despite treatment with base adequate to prevent acidosis some animals die, apparently in central nervous-system depression.

3. Despite the treatment with base most animals show retinal edema. In some this is confined to the perimacular region; in others it extends to the entire fundus; and in still others papilledema follows the fundus edema. Regression of symptoms is in reverse order: the disc clears first, then the peripheral fundus, the central area last of all.

Western Reserve University (6).

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Discussion

Dr. Zacharias Dische: I would like to ask a few questions concerning the parallelism between the effect of the formic acid and formaldehyde on retinal glycolysis and the effect on the retina. It was not quite clear to me which way the formaldehyde and the formic acid were applied. Was it intravenously or subcutaneously or some other way?

This has an important bearing, because obviously the concentration of these agents in the blood is of very great importance, particularly because the effects are reversible. In the methanol effect, which is irreversible, concentration has to reach a certain level for only a very short time, possibly. In the case of such reversible effects, the concentration which is in the blood must be sufficiently high during the duration of the effect.

It is well known that formaldehyde has a very high affinity to proteins, particularly blood proteins. It appears with terrific speed in the red cells by some combination with the proteins, so I think this is an important factor in any interpretation of these results.

The next question is whether there would be a dilution of your homogenates. I assume you used retinal homogenates of the cation. In this case I think the dilution which you had to go to in preparing the homogenates would be of great importance for the interpretation of these results.

I also wonder whether you did some experiments on the effect of respiration, because in general the respiration is much more sensitive to such toxic agents of a rather general nature, such as formaldehyde or formic acid, than is glycolysis. It would be interesting if you would care to point it out.

Dr. David G. Fleming: In connection with the methanol-formaldehyde-formic acid intoxication, did you consider using something like antabuse, which would dam up the oxidative mechanism of the methanol?

As far as I know, antabuse prevents the formaldehyde from being converted into formic acid, and there is an excess accumulation of formaldehyde. This would indicate whether your primary toxicity was from the formaldehyde in vitro or whether it was from the formic acid.

Dr. A. M. Potts (closing): The formaldehyde was given intravenously. We consider this adequate proof that, despite the great affinity of formaldehyde for tissues, it does reach the eye in sufficient concentrations to cause these effects. Whereas, we do not postulate that the concentration in molarity is as great in the eye as in the initial solution we do say that it is no greater than the amount given. It is probably much, much less, and still it has a dramatic effect.

This is also significant when one considers the possible site for potential formaldehyde formation from methanol in the animal. It means that one does not have to make formaldehyde in the eye in order for it to react with the eye, but that methanol could conceivably be oxidized in the liver and that sufficient formaldehyde could still reach the eye to cause significant eye effects.

The other materials were given by stomach tube largely because of the large volumes required. The methanol dose was tremendous, and was lethal in all cases. It is important to emphasize that these dosages of methanol and formate were huge in comparison to the minute formaldehyde dosage that achieved the same effect.

As far as inhibition of oxidation is concerned, we have published material on the subject previously. The work Dr. Praglin was describing was presented by us in Atlantic City in 1952. The effect on oxidation is considerably less than the effect on glycolysis with equal quantities of formaldehyde. The tissue concentration in all of those experiments was approximately 100 mg. of homogenate per Warburg vessel, which was 7.0 cc. total volume.

In regard to the question about antabuse, work on this by Dr. Gilger also was published, and was presented at an early meeting of this association. We found at the time that antabuse increased considerably the toxicity of methanol for small animals. We considered and we consider now that this upping of the toxicity by antabuse is due to an added formaldehyde factor superimposed on the ordinary narcotic effect of the alcohol. Antabuse in monkeys is something that we have not done, but it is on our schedule because it is highly important.

THE LIEBMANN EFFECT IN BINOCULAR PERCEPTION*

Preliminary studies

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When a colored figure and its neutral background are equally luminous and fixation is maintained at the center, the figure appears to lose its clarity and fade into the background.[1] It was thought that this phenomenon, known as the Liebmann effect, might with profit be subjected to quantitative and binocular studies.

Method

The main problem has been the construction of an adequate apparatus for the binocular study of the Liebmann effect.

Original apparatus:

The original apparatus consisted of three main parts: (1) screen, (2) observation post; (3) three-dimensional projector (fig. 1).

A large "frozen" acetate screen is viewed through two holes in which are placed Polaroid sheets. These Polaroid sheets have their axes of transmission at right angles to each other, arranged so the right and left eyes observe only their respective slides; thus the stimulus to each eye can be separately controlled.

The slides used in the three-dimensional projector utilize the isotropic property of cellophane. Cellophane has the property to rotate the plane of polarization of light through 90 degrees. In constructing the slides cellophane is sandwiched between two pieces of Polaroid. If the two external pieces of Polaroid are set with their planes of polarization 90 degrees from each other, light will be transmitted, since the cellophane will rotate the plane of polarization from the position of the first to that of the second Polaroid filter. If the two external pieces of Polaroid are set at 45 degrees to each other, 50 percent of the incident light is transmitted through them. Colored cellophane was used between the two pieces of Polaroid to produce the figure. This colored cellophane was rotated by visual match until it also transmitted nearly 50 percent of the incident light. Thus, both figure and ground were made as equally luminous as possible.

With this original appartus two variables were studied. In one set of slides the size of the figure was varied. Three equilateral red diamonds had visual angles of 16, 32, and 52 degrees, respectively. The second set of three slides varied the ground size while the figure was kept constant. The slide with the largest ground was the 100-percent slide. The other two slides had a ground size, 50 percent and 25 percent of the size of the largest one. The respective projected images were approximately 56 by 57 degrees, 41 by 44 degrees, and 25 by 31 degrees. The figure was a red cellophane circle which subtended an angle of 16 degrees.

The level of darkness in the experimental room was far below the photopic threshold and the subject was shielded from light escaping from the apparatus.

Subjects

There were six subjects of both sexes between the ages of 25 and 35 years. None wore glasses for reading and all but one reported never having had trouble with strabismus. This individual had had no trouble since childhood and her results on the following experiments did not differ

* From the Department of Ophthalmology, Indiana University School of Medicine. This investigation was supported by a research grant (B-86) from the National Institutes of Health, Public Health Service. We wish to acknowledge the advice and help of the Division of Optometry, Indiana University, especially that of Dr. Merrill J. Allen.

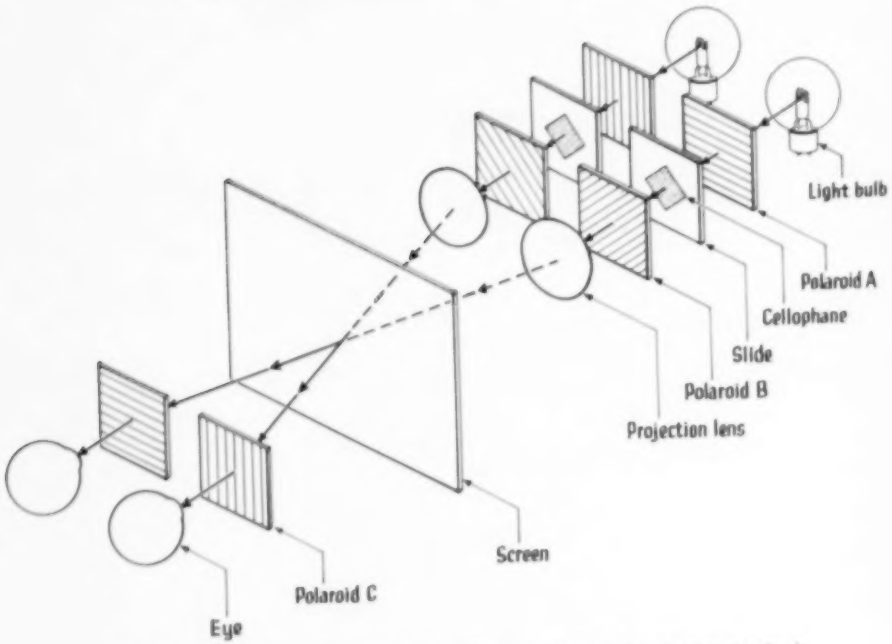

Fig. 1 (Orzack and Schlaegel). Schematic diagram, showing the path of polarized light from the light source to the eye.

significantly from those of the other subjects.

Procedure

Since the Liebmann effect was to be studied under binocular conditions, as in a stereoscope, it was necessary to check the fusion of the subjects. This was done with the Orthofusor.* If the subject could properly perceive the first three cards without difficulty and the last two with some difficulty, he was considered suitable. By the use of red goggles the subject was dark adapted for 20 minutes during which interval he was instructed that he would be required to fixate on the center of the figure, that the figure would change, and that he should report all such changes.

A test slide was shown for three minutes. During this time the subject's oral responses were recorded by the experimenter to test further his eligibility as a subject. After this trial presentation, the subject was told to put the goggles on again and relax his eyes. A five-minute interval was allowed between slides. During this period the subject was told that he would be shown more slides which would change as did the previous one. He was to press one telegraph key for partial changes and another key for total changes. These keys activated an Esterline-Angus recorder. Fixation in the center of the figure was again emphasized.

The subject was then shown the second practice slide, after which he was allowed to rest for five minutes. Next, the three stimulus slides were presented for five minutes each with five-minute intervals between them. After each presentation the subject was asked to report on what he had seen while viewing the slide.

Results

The responses were divided into three

* Manufactured by Bausch & Lomb Optical Company.

classes:

1. Complete disappearance of the figure.
2. Partial disappearance of the figure.
3. Reappearance of the figure.

We recorded when the disappearances and appearances occurred, how frequently they occurred over a period of five minutes, and how long the figure was present or absent.

Figure 2 demonstrates that the diamond figure disappears partially more frequently than it disappears completely. It also demonstrates that the larger the diamond the more frequently it completely disappears. Although there is a similar tendency for the diamond to disappear partially the larger it is, this was not statistically significant.

In Figure 3 we can see that the larger the diamond the longer it remains invisible. Partial disappearances last for longer times than total disappearances. Partial disappearances last for longer periods as the size of the diamond increases but this is not statistically significant.

In Figure 4 we can see that the diamond always disappears partially before it disappears completely. The larger the diamond the more quickly it disappears. Partial disappearances also occur more quickly but this is not statistically significant.

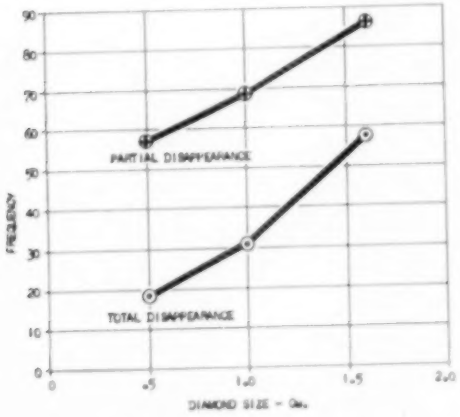

Fig. 2 (Orzack and Schlaegel). Frequency of partial and total disappearances during a five-minute period as a function of diamond size. The plotted points are the mean for six subjects.

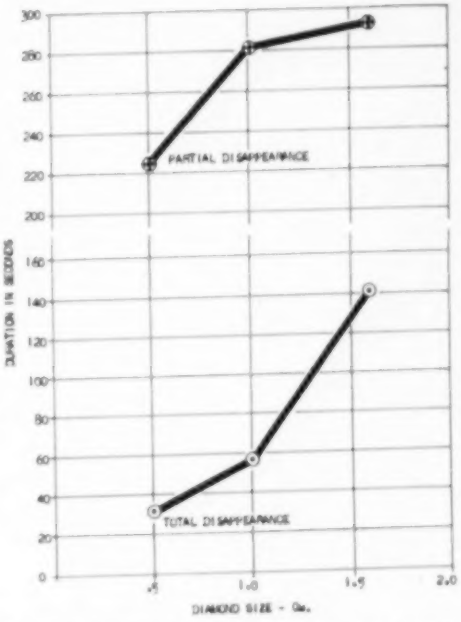

Fig. 3 (Orzack and Schlaegel). Duration of total and partial disappearances during a five-minute period as a function of diamond size. The plotted points are the mean for six subjects.

Summarizing the results of this first experiment, we can say that the Liebmann effect is enhanced by an increase in size of the figure. The larger the figure the more quickly and the more frequently it will disappear and it remains invisible for a longer period.

In our second experiment we used a red disc of constant size but varied the size of the ground. As the size of the ground is increased the figure disappears less frequently (fig. 5). As the size of the ground is increased the figure remains invisible or disappears totally for a shorter time, but partial disappearances tend to last for a longer time (fig. 6).

Comment

The enhancement of the Liebmann effect with increasing size of the figure may be explained by decreased visual acuity and

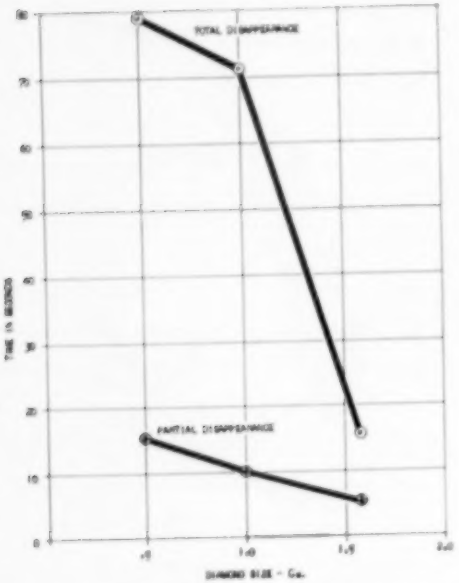

Fig. 4 (Orzack and Schlaegel). Time of first partial and total disappearance as a function of diamond size. Each point represents the mean for six subjects.

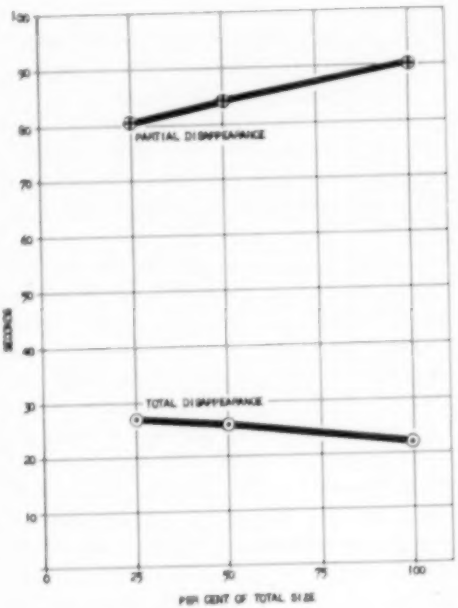

Fig. 6 (Orzack and Schlaegel). Durations of total and partial disappearances during a five-minute period are plotted as a function of the size of the field. Each point represents the mean for six subjects.

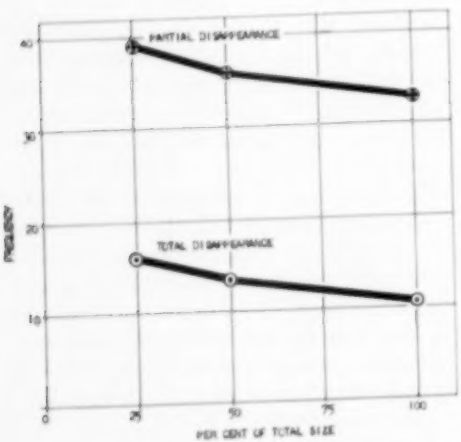

Fig. 5 (Orzack and Schlaegel). Frequencies of total and partial disappearances during a five-minute period are plotted as a function of the size of the field. Each point represents the mean for six subjects.

retinal adaptation.[2] With the larger diamond figures the subjects reported difficulty in seeing the entire figure at one time. It was difficult to perceive color in the outer portions of the figure. Since the border between the figure and ground is one of color, not intensity, and since visual acuity is basically an intensity discrimination, visual acuity is near zero. In addition, as a result of steady fixation in the center of the figure retinal adaptation or fatigue occurs. Both poor visual acuity and retinal adaptation work together to cause a disappearance of the figure. Since the visual acuity is reduced as the figure is made larger, the figure will disappear more quickly, more frequently, and will remain invisible for longer periods.

The lessening of the Liebmann effect by an increase in size of the ground may be explained by the principle of border inhibition. Fry and Bartley[3] discovered that an

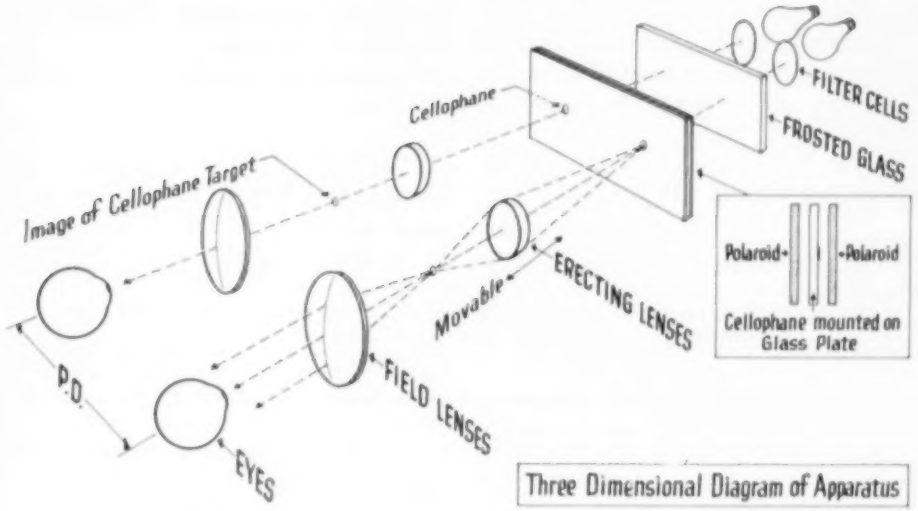

Fig. 7 (Orzack and Schlaegel). Three-dimensional diagram of apparatus.

outside border inhibits the discrimination of a test patch. With a large ground the subject sees only the inner border made by the figure and ground. The outside border of the field, while visible, is too peripheral to have much effect and therefore does not inhibit the borders of the figure. As a result only retinal adaptation is active in the production of the Liebmann effect. The influence of border inhibition is increased as the ground is made smaller. The figure disappears more frequently because the effect of border inhibition is added to that of retinal adaptation.

Present apparatus

The present apparatus was developed in an attempt to correct the following deficiencies in the original set-up: (1) The screen was not equally illuminated over its entire surface because the projector produced a hot spot in the center; (2) a change in the size of the figure or ground requires a series of slides. It has been impossible to make such a series identical except for the variable to be studied.

The present apparatus is diagrammed in Figure 7. The light source sends rays through frosted glass which disperses it evenly. The light then passes through Polaroid filters as before. Erecting lenses condense and transmit the light to field lenses which refract the light to parallel rays. Thus the image appears to be at infinity. The erecting lenses may be moved within a short space to produce a change in size of the image. Changes in luminosity are accomplished by neutral-tint Wratten filters placed in front of the light source.

The area behind the Polaroid filters is enclosed in a light-tight box, the cover of which serves as a shield for the light bulbs. Thus, no light other than that through the lenses is visible to the subject. The area visible to the subject is about 30 degrees in diameter, which is less than that of the original apparatus but still sufficient for a study of the variables in which we are interested.

Summary and conclusions

A figure and ground phenomenon known as the Liebmann effect was studied binocularly by using a stereoscopic projector and auxiliary filters.

The following tentative conclusions can

be drawn from this study:

1. The larger a diamond figure the more quickly it disappears, the more frequently it disappears, and the longer it remains invisible.

2. The larger the ground, the less frequently a disc figure disappears.

3. Partial disappearance invariably precedes total disappearance.

An improved apparatus will be used in future studies.

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Medical Center.

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Discussion

Dr. Gerhard A. Brecher: I was very much interested in this paper by Dr. Orzack and Dr. Schlaegel, and I think the work presented is a very fine contribution to an exact evaluation of the Liebmann effect.

There are two questions I would like to ask Dr. Schlaegel. First of all, when he presents his data in final form, might it be advisable to present the size of the figure as well as the ground in the angles of vision, instead of just size in centimeters or some other unit?

Secondly, I missed entirely seeing any connection between this phenomenon as described and binocular perception. Actually, I believe the same effects could be obtained, if I am not mistaken, in monocular vision. It is very important to stress this, because the title of the paper indicates a contribution to binocular perception, which I cannot see.

Dr. T. F. Schlaegel, Jr., (closing): The sizes in degrees were given, and I mentioned them hurriedly. Perhaps it would have been better to have shown them on the slide also, but they are in the final paper.

You are entirely correct about this not being a binocular study. In other words, the same thing would occur with naked vision or with one eye at a time. The main thing we have attempted to do is to develop an apparatus and to get started on this type of work. That was what took most of the time.

This is a preliminary report, and is so entitled. We feel we have it more or less set up now so that we can go ahead and do binocular studies in the future, and we feel that our main contribution is the apparatus and getting ready for making future studies along the lines of binocular perception.

THE BREAKDOWN OF GLUCOSE AND ITS PHOSPHORIC ESTERS IN THE BOVINE LENS CAPSULE*

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New York

It has been previously reported[1] that even after removal of the epithelium, the beef lens capsule in vitro breaks down ribose contained in its nucleotide fraction under aerobic as well as anaerobic conditions. As ribose in the nucleotides is substituted on its Carbon 5 by phosphoric acid, it was reasonable to assume that its breakdown follows the pathway which was established for the breakdown of ribose-5-phosphate[2] in other tissues. This pathway consists of the transformation of ribose-5-phosphate into a mixture of hexose-6-monophosphate and fructose-1, 6-diphosphate.

The fact that it was not possible to demonstrate an accumulation of these fructose esters during the breakdown of ribose was attributed to rapid metabolism of these esters by the lens capsule. These esters, on the other hand, are also intermediates in the aerobic and anaerobic breakdown of glucose in all tissues, and a priori it was reasonable to assume that glucose, which is present in the lens capsule, represents the normal metabolic substrate of the lens capsule.

In the present series of experiments, therefore, the ability of the lens capsule to metabolize glucose, glucose-6-phosphate, and fructose-1, 6-diphosphate (Harden-Young ester) under anaerobic and aerobic conditions was investigated. These investigations were extended also to lens capsule from which the epithelium was removed by scraping.

EXPERIMENTAL

MATERIAL AND GENERAL PROCEDURES

Cattle eyes from animals 18 to 30 months of age were obtained from the slaughter house. The eyes were removed a short time after killing of the animal, were transported packed in ice to the laboratory, and were immediately dissected. The preparation of the lens capsules and removal of the epithelium were carried out as previously reported.[1]

In the experiments in which the epithelium was not removed, the whole capsule was used after the fibers were removed as carefully as possible. In those experiments in which the epithelium was removed, only the anterior capsule was used.

Every capsule was spread on a microscopic slide which, in order to facilitate better visibility, had been blackened with charcoaled paraffin. The capsules were divided into two equal parts as accurately as possible. One half of each capsule served as a control for the determination of the initial value of sugar, while the other half was transferred into a previously weighed standard Warburg vessel containing one cc. of Kreb's Ringer solution. Five to 10 halves in pooled batches weighing 100 to 200 mg. were used for every experiment.

In experiments in which no substrate was added to the capsule, the control halves were transferred to centrifuge tubes containing one cc. of Kreb's Ringer solution and immediately deproteinized by adding a volume of 100-percent trichloracetic acid sufficient to make the final concentration five percent, while the experimental samples were incubated for two hours in a water bath at 37°C. under vigorous shaking in an atmosphere of pure oxygen or nitrogen, which gases were previously bubbled through the Kreb's Ringer solution used for the experiment. At the end of this time, the experiment was

* From the Department of Ophthalmology, Columbia University College of Physicians and Surgeons, and the Institute of Ophthalmology, Presbyterian Hospital. This work was performed under Contract AT-30-1-Gen. 70 of the Atomic Energy Commission.

interrupted by the addition of 100-percent trichloracetic acid to bring the final concentration to five percent and the sample was left standing overnight.

Next, an aliquot of the trichloracetic-acid filtrate in the Warburg manometers was centrifuged, together with the control sample. The protein residue in the Warburg vessels was then rinsed with five-percent trichloracetic acid into the corresponding residue of the experimental sample in the centrifuge tube, centrifuged again and nitrogen determinations were made from the total residue of this sample as well as of th. control. In some experiments substrate was added to the suspension fluid of the experimental sample. In these cases, the control sample (without any added substrate) was not deproteinized immediately, but was incubated under the same conditions as the experimental sample.

Analytic procedures

Glucose and glucose-6-phosphate were determined by two different methods, namely the anthrone reaction[3] and the cysteine sulfuric acid reaction for hexoses.[4] These two reactions were in satisfactory agreement. Fructose-1, 6-diphosphate was determined as fructose by the cysteine carbazole reaction previously described,[5] and as hexose by the cysteine sulfuric acid reaction of hexoses. Duplicates were taken for every determination and an internal standard was used throughout. Lactic-acid determinations were carried out by the Barker-Summerson method,[6] nitrogen was determined by micro-Kjeldahl, and inorganic and pyrophosphate P by the method of King.[7]

Results

Breakdown of glucose- and fructose-phosphates by the lens capsule

In a first series of experiments, the breakdown of glucose-6-phosphate, and of fructose-1, 6-diphosphate have been examined. These sugar esters were added to the suspension fluid in amounts which corresponded to 40 μg. of glucose and 30 μg. of fructose respectively. Under these conditions the loss of sugar caused a deviation of readings in the determination which was optimal as far as the accuracy of the determination was concerned.

The results of these experiments are listed in Table 1. As can be seen, the lens capsules break down both esters with a similar speed, and no difference in the rate was observed between aerobic and anaerobic experiments. It can be also seen from the table (experiment 26) that addition of monobromoacetate, in concentration of M/100 and M/40 NaF, inhibited almost completely the breakdown of the esters. This finding indicates that the breakdown follows the pathway known from other tissues, and involves formation of triosephosphate and its oxidation by triosephosphate dehydrogenase.

The determination of lactic acid in these experiments did not provide a definite answer as to the end-product of the breakdown of the sugar esters because of the high initial level of lactic acid in the lens capsule which, in our experiments, was about 100 gamma/100 mg. wet weight of the capsule. Under these conditions the accumulation of lactic acid from the sugar esters could not amount to more than 10 percent of the initial value, and it was impossible to obtain statistically significant results for the amount of lactic acid produced from these esters.

As can be seen from experiment 53, hexose-6-phosphate was broken down by the capsule even after the epithelium was scraped off as carefully as possible. The rate of the breakdown was somewhat lower than the average rate from four experiments with intact epithelium. It was, however, higher than the lowest values in the latter series of experiments.

As can be seen from Table 2, fructose-1, 6-diphosphate is broken down by the capsule at about the same rate as glucose-6-phosphate, as far as the breakdown of the total hexose is concerned. It will be noted, how-

TABLE 1

BREAKDOWN OF GLUCOSE-6-PHOSPHATE IN BEEF LENS CAPSULES INCUBATED AT 37° C.

Experiment No.	Weight of Lens Capsule (in mg.)	Gas Phase in Warburg Vessel	Time of Incubation (in hr.)	Substrate Added per μg./gm. Capsule	Total Hexose per μg./gm. Capsule	Amount of Hexose used up per μg./gm. Capsule
17	341.0 321.0	— O	0 2	Gl-6-Phos 124 Gl-6-Phos	345 240	— 112
23	312.0 315.0	— N	0 2	Gl-6-Phos 167 Gl-6-Phos	338 157	— 184
53*	260.3 252.0	— N	0 2	Gl-6-Phos 150 Gl-6-Phos	384 288	— 84
19	273.0 262.5	O O	2 2	0 Gl-6-Phos 145	310 236	— 62
21	305.0 293.0	O O	2 2	0 Gl-6-Phos 130	387 309	— 63
18	420.5 421.0	N O	2 2	Gl-6-Phos 95 Gl-6-Phos 95	270 270	— 0
26	396.0 393	Air Air	2 2	Gl-6-Phos 100 Gl-6-Phos 100 (+M/100 Bromo-acetate and M/40 NaF)	180 300	— —

* Epithelium scraped off.

ever, from the last column of the table that the amount of fructose used up was smaller than the amount of total hexose. This could mean that in these experiments the lens capsule was also able to utilize its own glucose.

BREAKDOWN OF GLUCOSE IN THE LENS CAPSULE

The concentration of glucose in the lens capsule varies from 20 to 40 mg. percent. These values are lower than those found in the aqueous humor and in the lens fibers. This difference may be due to changes during the preparation. When the isolated lens capsule was incubated, no significant decrease of glucose in two hours could be observed in five out of eight experiments, nor was any significant breakdown observed

TABLE 2

BREAKDOWN OF FRUCTOSE-1,6-DIPHOSPHATE IN BEEF LENS CAPSULES INCUBATED AT 38°C.

Experiment No.	Weight of Lens Capsule (in mg.)	Gas Phase in Warburg Vessel	Time of Incubation (in hr.)	Substrate Added per μg./gm. Capsule	Total Hexose per μg./gm. Capsule	Amount of Hexose Used up per μg./gm. Capsule	Fructose per μg./gm. Capsule
13	387.0 360.0	— O	0 2	Fr-diphos 83 Fr-diphos	307 157	— 122	143 83
15	399.5 379.0	— O	0 2	Fr-diphos 87 Fr-diphos	330 230	— 83	137 90.3
24	293.0 280.0	Air Air	2 2	Fr-diphos 140 Fr-diphos 140 (+M/1000 Bromo-acetate+M/40 NaF)	185 290	— —	— —

TABLE 3

BREAKDOWN OF GLUCOSE BY BEEF LENS CAPSULES

Experiment No.	Wet Weight of Capsules (in mg.)	Gas Phase	Time of Incubation (in hr.)	Glucose in μg./gm. Capsule	Glucose Used up in μg./gm. Capsule
3 a)	288.3 331.5	O_2	0 2	319 297	71
b)	240.8 249.5	O_2	0 2	324 336	0
4 a)	358.0 348.0	O_2	0 2	402 375	15
b)	218.0 243.0	O_2	0 2	380 380	0
5 a)	271.0 316.5	N_2	0 2	356 312	52
b)	223.0 278.0	N_2	0 2	336 269	96
6 a)	302.0 215.0	N_2	0 2	413 435	0
b)	328.0 339.5	N_2	0 2	387 412	0

when glucose was added to the suspension fluid in such amounts that its final concentration was about 30 mg. percent (table 3).

The fact that the phosphoric esters of glucose, but not glucose itself, were attacked by the lens capsule in every experiment suggested that the inability of the lens capsule to metabolize glucose in the majority of cases may be due to variations in its ability to phosphorylate glucose to glucose-6-phosphate. This suggestion was supported by the fact that the adenosine triphosphate which is present in the capsule in a concentration of about M/2,000, decreases rapidly during the incubation at 37°C. The results in Table 4 show that after this interval of time the concentration of adenosine triphosphate drops to 20 to 30 percent of the original value.

To test the validity of this assumption, we carried out a series of experiments in which adenosine triphosphate in the form of its neutral sodium salt was added to the suspension fluid at the beginning of the experiment to one batch of halves of capsules, while to the other batch which contained only glucose, no adenosine triphosphate was added. These samples were incubated at 37°C. for two hours and the glucose content of both was determined.

As may be seen from Table 5, in four out of five of these experiments the amount of glucose present at the end of the experiment in samples to which adenosine triphosphate had been added, was 20 to 35 percent lower than in samples without adenosine triphosphate. These results support our assumption that the seeming inability of the capsule to metabolize glucose is only an apparent one due to the rapid dephosphorylation of adenosine triphosphate in the stripped-off capsule which depresses this phosphate carrier to a level at which the significant phosphorylation of glucose can no longer take place.

When the same experiments were repeated with capsules from which the epithelium was removed, breakdown of glucose in presence of adenosine triphosphate could be still observed, although here again the breakdown was only about half of the average found in presence of intact epithelium.

TABLE 4

INFLUENCE OF INCUBATION IN PRESENCE AND ABSENCE OF GLUCOSE-6-PHOSPHATE ON THE INORGANIC AND PYROPHOSPHATE P OF BEEF LENS CAPSULES AT 38°C.

Experiment No.	Wet Weight of Capsule (in mg.)	Time of Incubation (in hr.)	Substrate Added per μg./gm. Capsule	Inorganic P per μg./gm. Capsule	P Hydrolyzed in 10 min. by N H_2SO_4 per μg./gm. Capsule
29	372.5	0	Gl-6-Phos 100	94	46
	633.0	2	Gl-6-Phos	149	15
28	440.5	0	0	93	30
	493.0	2	0	128	11
30	432.0	1	Gl-6-Phos 110	129	15
	438.0	1	0	130	11

DISCUSSION

Our experiments indicate that the lens capsule of beef is able to metabolize glucose under anaerobic conditions; there appears no difference, at least in vitro, in the turnover of glucose in presence of oxygen or nitrogen. As the lens capsule in two hours' heating was found to utilize about 10 μl. of oxygen under the conditions of our experiment, our data indicate that glucose is predominantly glycolyzed and not oxidized.

Although it was not possible to establish an increase in lactic acid during the breakdown of glucose and glucose esters, there can be little doubt that the pathway of the breakdown is the usual glycolytic one; this is indicated by the speed with which hexose-6-phosphate and fructose-1, 6-diphosphate, the usual intermediates in the glycolytic breakdown of glucose, are metabolized, and by the complete inhibition of the breakdown of these esters by monobromoacetic acid in combination with NaF.

Finally, the fact that the metabolism of glucose by the capsule requires an adequate level of adenosine triphosphate, indicates clearly that phosphorylation of glucose to hexose-6-phosphate is the initial step in its breakdown by the capsule. The observation that hexose-6-phosphate is metabolized even

TABLE 5

INFLUENCE OF ADDITION OF ADENOSINE TRIPHOSPHATE ON THE BREAKDOWN OF GLUCOSE IN BEEF LENS CAPSULES

Experiment No.	Wet Weight of Capsule (in mg.)	Gas Phase	Time of Incubation (in hr.)	Substance Added in μg./gm. Capsule	Total Glucose in μg./gm. Capsule	Glucose Used up in μg./gm. Capsule
36	441.5	Air	2	ATP 1.5	151	
	491.5	Air	2	0	250	74
37	431.0	N_2	3½	ATP 1.5	374	
	466.0	N_2	3½	0	238	136
34*	344.0	Air	2	Glucose 0.2 ATP 1.5	268	
	354.0	Air	2	0 ATP	378	102
35	353.0	Air	2	Glucose 0.2 ATP 1.5	258	
	352.0	Air	2	0 ATP	253	0
52*	410.0	Air	2	ATP 1.5	304	
	400	Air	2	0	370	76

* In these experiments glucose was determined by the anthrone as well as the cysteine reaction.

without the addition of adenosine triphosphate does not invalidate this conclusion as the phosphorylation of fructose-6-phosphate to fructose-1, 6-diphosphate by adenosine triphosphate in other tissues (non-nucleated and nucleated red cells) was shown to proceed much faster than the phosphorylation of glucose to the equilibrium mixture of fructose and glucose-6-phosphate, and to go on with sufficient speed even in the presence of catalytic amounts of adenosine triphosphate. In this respect, therefore, the lens capsule does not differ essentially from other tissues.

The rapid breakdown of hexose esters suggested the possibility that the ribose-5-phosphate present in the capsule in nucleotide form may be metabolized in a similar way, as was shown to be the case in other tissues such as blood and liver. This pathway would involve, as was pointed out previously, the formation of heptulose-7-phosphate, triosephosphate, and hexose-6-phosphate as intermediates. However, attempts to demonstrate an accumulation of either of these intermediates during the incubation of the bovine lens capsules were negative.

Furthermore, addition of ribose-5-phosphate or adenosine, which in other tissues is rapidly phosphorylated to ribose-5-phosphate, did not cause any increase in the turnover of ribose. Hexose-6-phosphate added to the capsule is easily metabolized and, therefore, must easily penetrate the capsule. It seemed therefore improbable, although not impossible, that this failure to obtain a breakdown of added ribose-5-phosphate or adenosine was due to the inability of these compounds to penetrate into the capsule.

The possibility had to be considered that the enzyme system metabolizing ribose-5-phosphate is saturated already at the low concentrations of this ester present in the capsule. This would explain the failure in demonstrating any significant increase in the turnover of ribose by added ribose-5-phosphate or adenosine. If such were the case, the interruption of the glycolytic breakdown of hexose-6-phosphate by bromoacetic acid, which does not inhibit the disappearance of ribose-5-phosphate, should lead to an accumulation of either heptulose phosphate or herose phosphate. However, the most sensitive test for heptulose with cysteine and sulfuric acid,[8] and for fructose phosphate with cysteine and carbazole and sulfuric acid, failed to detect any amount of these compounds formed from the ribose of the lens capsule nucleotides in presence of bromoacetate. Therefore, the breakdown of ribose-5-phosphate in the lens capsule probably proceeds on a different pathway. This requires further investigation.

Our observations on the disappearance of adenosine triphosphate in the capsule raised a question of the extent to which the metabolism in vitro of this tissue resembles, insofar as turnover is concerned, its metabolic activities in vivo. This rapid disappearance of adenosine triphosphate could be, in the first place, due to an increase in the activity of the dephosphorylating enzymes, probably adenosine triphosphatase. An increase in the dephosphorylation of adenosine triphosphate after injury is quite common in all tissues and reaches almost explosive dimensions in such tissues as heart, muscle, or nucleated red cells.

The other possibility is that adenosine triphosphate is present in a steady-state due to a dynamic equilibrium between the breakdown by adenosine triphosphatase or phosphate-transferring enzymes and the resynthesis brought about by phosphorylation coupled with glycolysis or respiration. In this case the rapid destruction of adenosine triphosphate in vitro would be due to a significant decrease in the metabolic rate or its coupling with adenosine triphosphate synthesis as a result of stripping off the capsule. This decrease in metabolic rate could concern only the respiratory metabolism,

as the efficiency of this metabolism, as far as phosphorylation is concerned, is several times higher than that of the glycolytic process.

In either case we would have to assume, therefore, that the separation of the capsule from the lens fibers causes severe damage to its structural elements. In this respect again the lens capsule shows the properties of a living organ highly sensitive to environmental changes.

In view of these considerations concerning adenosine triphosphate, the metabolism of the nucleotides in the lens capsule appears to be most probably a post mortal phenomenon depending upon the splitting of adenosine triphosphate, and perhaps also of coenzymes 1 and 2. As long as these nucleotides are not split, the adenosine-5-phosphate which can serve as a source of ribose-5-phosphate cannot be split to deliver this latter ester. We can assume, therefore, that in vivo the ribose-5-phosphate in the nucleotides is either metabolized not at all or to a much lesser degree, depending upon the continuous presence of small amounts of free adenosine-5-phosphate, which obviously cannot be determined at the present time.

Summary

1. The adenosine triphosphate in the lens capsule of beef is rapidly dephosphorylated after the capsule is stripped off and incubated at 37°C.

2. Anaerobic breakdown of glucose by the lens capsule can be observed reliably only in presence of sufficient amounts of adenosine triphosphate.

3. Removal of the epithelium depresses the glucose turnover but does not abolish it.

4. Glucose-6- and fructose-1, 6-diphosphate are anaerobically broken down by the lens capsule even without addition of adenosine triphosphate.

5. Ribose-5-phosphate and adenosine added to the lens capsule are not metabolized.

6. The significance of these findings for the interpretation of the capsular metabolism in vivo is discussed.

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ANAEROBIC CARBOHYDRATE METABOLISM OF THE CRYSTALLINE LENS*

I. Glucose and glucose-6-phosphate

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Kronfeld and Bothman[1] in 1928 first demonstrated the ability of the crystalline lens to produce lactic acid from glucose. Since then, fragmentary evidence has accumulated suggesting that lens glycolysis involves intermediary phosphorylation and the participation of phosphate esters.

Süllmann[2] in 1937 demonstrated the ability of bovine lens to form acid soluble organic phosphates with the disappearance of inorganic phosphate in the presence of various sugars. One year later Weekers and Süllmann[3] observed a 10-percent increase in lactic-acid formation by bovine lens in the presence of added inorganic phosphate. However, no stoichiometric relationship could be established. Increased glycolysis also resulted from the addition of yeast hexokinase, and hexosemonophosphate and hexosediphosphate were as effective as glucose in producing lactic acid.

Müller[4] in 1936 showed an increase in alkali saponifiable phosphate when bovine lens was incubated with fructose diphosphate. More recently Palm[5] and Müller and Kleifeld[6] reported the rapid incorporation of P^{32} into acid soluble organic phosphate of the animal lens.

Nordmann and Mandel[7] detected in the lenses of cattle, rabbits, and rats practically all of the phosphate esters predicted from the Embden-Meyerhof scheme of carbohydrate metabolism. The work of Leinfelder and Christiansen[8] established the basic enzymatic nature of lens glycolysis by showing it to be a heat labile system.

Our knowledge of the carbohydrate metabolism of the crystalline lens is, indeed, very meager. Whether the lens, like all other mammalian tissues studied, behaves metabolically in accordance with the classical Embden-Meyerhof scheme remains to be established.

These investigations were undertaken to elucidate the metabolic pathway and to establish the intermediary reactions and enzyme systems involved in the anaerobic utilization of carbohydrates by the crystalline lens. The results obtained shall comprise a basis for the comparative evaluation of the metabolic characteristics of cataractous lenses. It is hoped that such a comparative study might shed some light on the biochemical mechanism of the development of lenticular opacities.

Materials

The following chemicals were purchased from Schwarz laboratories: barium glucose-6-phosphate, barium fructose-6-phosphate, dipotassium glucose-1-phosphate, adenosine-5-phosphoric acid, barium adenosine diphosphate, and diphosphopyridine nucleotide, chromatographically pure. Disodium adenosine triphosphate and hexokinase, dry lyophilized powder, were products of the Pabst laboratories. Glucose was Merck's anhydrous. Adenosine triphosphatase was prepared according to the method of Bailey.[9]

Procedures

Solutions of the barium salts of the phosphate esters were prepared by precipitating the barium ions in dilute HCl at 0°C. with 0.5M K_2SO_4, neutralizing with dilute NaOH, and removing the $BaSO_4$ by centrifugation and decantation. All solutions wherever possible were adjusted to pH 7.4.

* From the Research Department, Wills Eye Hospital. This work was supported in part by a grant-in-aid from the National Council to Combat Blindness.

Phosphate was determined by the method of Fiske and Subbarow[10] as modified by Lohmann and Jendrassik.[11] Labile phosphorus (7'P) was determined according to the procedure of Lohmann[12] at 100°C. and in N HCl.

Lactic acid* was determined according to Barker and Summerson[13] as described by Umbreit.[14] Adenosine-triphosphatase activity was assayed in the presence of 1.85 ml. 0.1N tris-(hydroxymethyl) amino methane buffer, pH 7.4; 0.15 ml. 0.018N $CaCl_2$; 0.5 ml. adenosine triphosphate solution containing 387 μg. 7'P. At 37°C. 0.5 ml. of enzyme solution liberated 150 μg. of inorganic phosphate in 10 minutes.

Preparation of lens extract

Colored rabbits between 20 and 30 days of age and weighing between 0.75 and 1.0 lb. were killed by the injection of one to two ml. of veterinary nembutal directly into the heart. Both eyes of each animal were enucleated immediately after death and placed in a beaker surrounded with ice.

The lenses were exposed by dissecting through the sclera, as described by Kinsey,[15] and removed free of vitreous humor and extraneous tissue as follows, care being exercised to avoid touching the lens tissue with any instrument: while the lens was in place atop the cornea, vitreous humor was drawn off by aspiration through a capillary tube connected to a H_2O aspirator. With a small muscle hook the zonular attachments were severed leaving the lens attached only in one narrow spot. By carefully drawing the scleral flaps over the cornea the lens may be suspended by the attached zonules. Any residual aqueous or vitreous humor usually collected as a drop on the lower tip of the lens and could be removed by touching it with gauze. The remaining zonular attachment was cut permitting the lens to drop into a chilled beaker. The entire procedure of removing the lens from an enucleated eye usually required not more than two to three minutes.

The weighed lenses (average weight per lens, 120 to 150 mg.) were transferred to a chilled Potter-Elvehjem homogenizer equipped with a smooth pestle† and sufficient isotonic KCl (0.154M) and $NaHCO_3$ (0.154M) were added to give a final concentration of one lens/0.5 ml. total volume, and a pH between 7.5 and 8.0.‡

Homogenization§ was conducted in the cold and was completed in about one minute with complete disintegration of the lens tissue. The homogenate was centrifuged at 4,000 rpm at 0°C. in an International Refrigerated Centrifuge, Model PR-1, for 10 minutes. The resultant opalescent supernatant was free of cellular debris when examined microscopically.

Experimental

All incubations were conducted in a Warburg apparatus at 37°C., initial pH of 7.4 to 7.6, and in a total liquid volume of 1.2 to 1.7 ml. All vessels were gassed at room temperature with 95-percent N_2 to 5-percent CO_2 for 10 minutes and equilibrated in the thermostatic bath for 10 minutes, during which time all glass-stoppered joints were tightened. The manometers were set at 15 and the contents of the side-arm tipped in at 0 minutes, unless otherwise noted. Controls for zero minute values, as well as suit-

* The source of $Ca(OH)_2$ was found to be of critical importance. Baker's, both U.S.P. and Analytical Reagent grade S, gave inaccurate and nonreproducible results. Instead of the characteristic blue or violet color, a green coloration formed with these materials. $Ca(OH)_2$ from Merck, Mallinckrodt, Harshaw, and Fisher were all equally satisfactory.

† Lens tissue was not completely disintegrated when a pestle with grooves or teeth was used.

‡ The dry weight content per lens was 20 percent. Thus, for example, 20 lenses, average weight per lens, 138 mg., contained 1.97 ml. H_2O. To this was added 6.69 ml. KCl and 1.34 ml. $NaHCO_3$ for a total volume of 10.0 ml.

§ Objectionable foaming was avoided by not lifting the pestle out of the mixture during the homogenization. After homogenization was complete, the pestle was slowly removed from the mixture.

able blanks, were run simultaneously in the given experiment.

Reactions were stopped or deproteinized with 0.2 ml. 40-percent trichloracetic acid either added from the side arm or pipetted into the flask that was removed and chilled at the end of the incubation. In either case the contents of the flask were made up to 10.0 ml. with five-percent trichloracetic acid, the protein precipitate thoroughly broken up with a glass rod, and centrifuged at 0°C. for 10 minutes at 4,000 rpm.

The clear colorless supernatant was used for analyses. Wherever extract values were necessary an aliquot of the original extract was treated similarly.

Results

All values are calculated on the basis of 100 mg. dry weight of lens tissue. Basic to the concept of a phosphorylating mechanism is the disappearance of inorganic phosphate in the progress of the reaction and the interdependency of the two phenomena. This relationship is strikingly demonstrated in Figure 1 and in Table 1. Thus, while the rate of glycolysis, as measured by the rate of evolution of CO_2 from $NaHCO_3$ buffer, slowed down after 40 minutes in the absence of added inorganic phosphate,* the rate was maintained constant for at least 120 minutes, when sufficient inorganic phosphate was present at the beginning of the reaction.

* The concentration of inorganic phosphate per ml. of lens extract was from 0.4 to 0.5 μM.

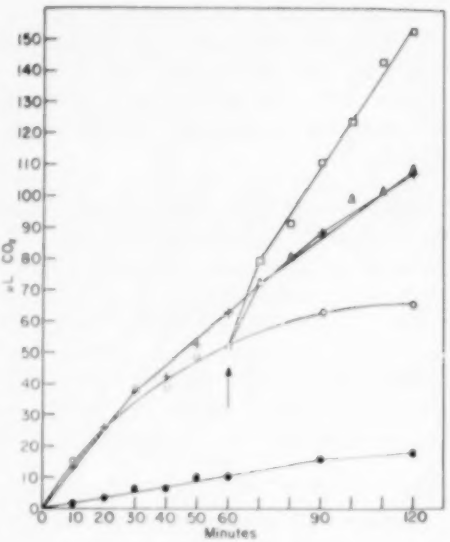

Fig. 1 (Green, Bocher, and Leopold). *The effect of phosphate upon glycolysis.*

The main compartment of each flask, except the blank, contained 1.11×10^{-2}M glucose and 1.0 ml. of lens extract equivalent to two lenses (dry weight, 85.8 mg.). T.V. 1.1 ml. In addition: —●—●—, blank, containing 0.1 ml. H_2O; —○—○—; 0.1 ml. H_2O; —+—+— 0.1 ml. 0.1M PO_4 solution (4.2×10^{-2}M. final concentration); 0.1 ml. 0.1M. PO_4 solution added at 60′ as indicated by the arrow (final concentration, 4.0×10^{-2}M in a total volume of 1.3 ml.); —□—□—, 0.1 ml. A_sO_4 solution added at 60′ as indicated by the arrow (final concentration, 2.08×10^{-2}M in a total volume of 1.3 ml.).

The same effect was achieved by tipping in from the side-arm of the vessel additional inorganic phosphate after the reac-

TABLE 1

Correlation of lactic acid formation with CO_2 evolution and inorganic phosphate disappearance

	μ Moles in 90 minutes		
Substrate	CO_2	Lactic Acid	Inorganic Phosphate
Glucose	3.46	1.00	0.89
Glucose + PO_4	4.72	4.52	3.70
Glucose + PO_4 (60′)	5.20	5.90	—
Glucose + ASO_4 (60′)	6.80	7.00	—
Blank	1.09	0.46	+0.90

The same conditions as in Figure 1.

tion had slowed down. This addition, as indicated by the arrow in Figure 1, restored the glycolysis to its original rate. The effect of the addition of arsenate was even more pronounced and confirmed the fact that the limiting factor under these conditions was inorganic phosphate.*

Since the evolution of CO_2 from $NaHCO_3$ is not always an accurate measure of the extent of production of lactic acid, the latter was determined chemically. It is clear from Table 1 that a quantitative relationship obtains between the CO_2 evolution and lactic acid formation when the reaction mixture is adequately fortified with inorganic phosphate; on the other hand, in the absence of added inorganic phosphate much more CO_2 (3.4 times) than lactic acid is formed.† It is also clear from Table 1 that the formation of lactic acid is correlated with the disappearance of inorganic phosphate.

When glucose-6-phosphate or fructose-6-phosphate is substituted for glucose as substrate, more than a doubling of the initial rate of formation of lactic acid occurred and an overall increase in 120 minutes of almost two-fold resulted. This is shown in Figure 2. This difference in capacity of the two substrates to produce lactic acid indicated that the formation of hexosemonophosphate‡ from glucose was a rate limiting factor. Assuming that glucose is converted to glucose-6-phosphate by the lens according to the classical reaction,

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-PO}_4 + \text{ADP},$$

this difference could be attributed either to an insufficient concentration of adenosine triphosphate or of hexokinase, or to both. Accordingly, it was found that the addition of 5.1 μM adenosine triphosphate had no stimulatory effect, while the addition of 1.0 mg. of hexokinase caused a 10-fold increase in rate and more than a five-fold increase in the overall production of lactic acid in 120 minutes (fig. 2). A similar proportional increase in lactic acid production was also observed when hexokinase was added to the control in the absence of exogenous substrate.

The observation that the formation of lactic acid was faster and greater in the presence of glucose and hexokinase than with glucose-6-phosphate as substrate, and

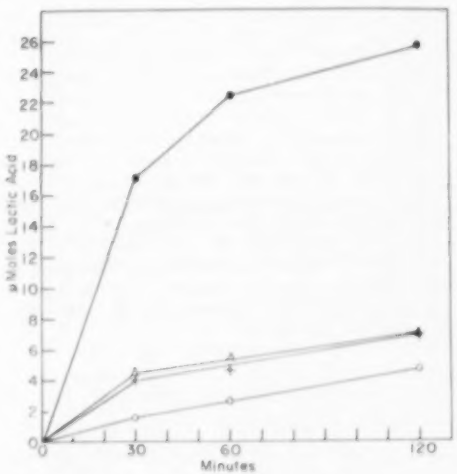

FIG. 2 (Green, Bocher, and Leopold). *Formation of lactic acid.*

Main compartment: 0.5 ml. extract, 0.5 ml. isotonic KCl-$NaHCO_3$, 0.1 ml. H_2O. (In the experiment with glucose alone 1.0 ml. of extract was used with no additional KCl-$NaHCO_3$.) Side-arm: 0.1 ml. glucose (final concentration, 1.11×10^{-2}M), 0.15 ml. 0.1M PO_4 (final concentration, 1×10^{-2}M), 0.05 ml. 0.031 N $NaHCO_3$, 0.1 ml. Hexokinase (1 mg. of crystalline material), 0.1 ml. glucose-6 or fructose-6-phosphate (final concentration, 6.67×10^{-3}M). Side-arm contents tipped in at 0 minutes. T.V., 1.5 ml. —○—○—, glucose; —+—+— fructose-6-phosphate; —Δ—Δ—, glucose-6-phosphate; —●—●—, glucose and hexokinase.

* Meyerhof[10] has shown that the addition of arsenate to yeast and muscle extracts permitted glycolysis to proceed without the accompanying uptake of inorganic phosphate.

† This is probably due to the Harden-Young effect[11] which may occur when the supply of inorganic phosphate is exhausted and results in the accumulation of acid equivalents (hexose mono- and diphosphate) other than lactic acid.

‡ Glucose-1-phosphate was equally effective.

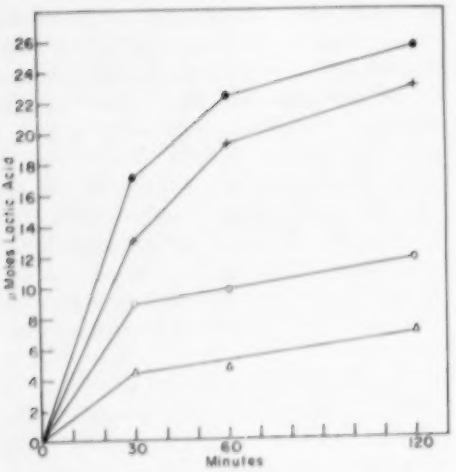

Fig. 3 (Green, Bocher, and Leopold). *Formation of lactic acid.*

Main compartment: 0.5 ml. extract, 0.5 ml. isotonic KCl-$NaHCO_3$ and 0.1 ml. H_2O. Side-arm: 0.1 ml. glucose (final concentration, 1.11×10^{-3}M); 0.1 ml. 0.1M PO_4 (final concentration, 1×10^{-3}M); 0.1 ml. hexokinase (1 mg. crystalline material); 0.05 ml. 0.031 N $NaHCO_3$; 0.1 ml. fructose-6- or glucose-6-phosphate (final concentration, 6.67×10^{-4}M); 0.1 ml. adenosine diphosphate (final concentration, 3.33×10^{-4}M); 0.1 ml. adenosine monophosphate (final concentration, 6.67×10^{-4}M). Side-arm contents tipped in at 0 minutes. T.V., 1.5 ml. —Δ—Δ—, glucose-6- or fructose-6-phosphate; —○—○—, fructose-6-phosphate and adenosine diphosphate; —+—+—, glucose-6- or fructose-6-phosphate and adenosine monophosphate; —●—●—, glucose and hexokinase.

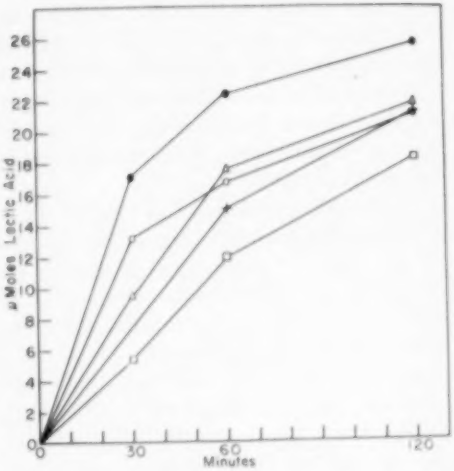

Fig. 4 (Green, Bocher, and Leopold). *Formation of lactic acid.*

Main compartment: 0.5 ml. extract, 0.5 ml. isotonic KCl-$NaHCO_3$, 0.1 ml. H_2O. Side-arm: 0.1 ml. glucose (final concentration, 1.11×10^{-3}M); 0.1 ml. hexokinase (1 mg. crystalline material); 0.15 ml. 0.1 M PO_4 (final concentration, 1×10^{-3}M); 0.05 ml. 0.031 N $NaHCO_3$; 0.1 ml. adenosine triphosphate (final concentration, 6.26×10^{-4}M); 0.1 ml. adenosine diphosphate (final concentration, 6.26×10^{-4}M); 0.1 ml. adenosine monophosphate (final concentration, 6.26×10^{-4}M). Side-arm contents tipped in at 0 minutes. T.V., 1.6 ml. —●—●—, glucose and hexokinase; —+—+—, glucose, hexokinase and adenosine triphosphate; —Δ—Δ—, glucose, hexokinase, and adenosine diphosphate; —○—○—, glucose, hexokinase and adenosine monophosphate; —□—□—, glucose, hexokinase, adenosine monophosphate, and adenosine triphosphate.

that the lens extract contained enough adenosine triphosphate to saturate the enzyme, indicated that the adenosine diphosphate formed in the reaction played an important role. Indeed, as shown in Figure 3, the addition of adenosine diphosphate or adenosine monophosphate to the glucose-6-phosphate or fructose-6-phosphate system caused a marked increase in the formation of lactic acid approaching the value obtained by glucose and hexokinase.

The system glucose and hexokinase, on the other hand, is somewhat inhibited by the presence of added adenosine monophosphate and adenosine diphosphate as well as by adenosine triphosphate (fig. 4).

Relationship of lactic acid production with CO_2 evolution

In an actively metabolizing system characterized by a well-balanced interplay of the glycolytic enzymes, the amount of CO_2 evolved from a bicarbonate buffer is quantitatively proportional to the amount of lactic acid formed. Whenever other acids besides lactic acid are being produced, or, conversely, if acid equivalents are being reduced, the evolution of CO_2 does not give an accurate measure of the extent of lactic acid formation. Under these conditions in order to measure the rate and extent of glycolysis it is necessary to determine the lactic

acid chemically. From an examination of the relationship between lactic acid production and CO_2 evolution one may gain some insight into the mechanics and enzymic properties of the system.

In the experiments with glucose without added phosphate the CO_2 evolution greatly exceeds the formation of lactic acid, while in the presence of added phosphate the two values are in good agreement. These observations, together with the fact that the addition of phosphate, arsenate, or myosin adenosine triphosphatase, after the reaction had slowed down, resulted in a marked increase in lactic acid production, suggested the operation of the Harden-Young effect. This is characterized, in this case, by the exhaustion of inorganic phosphate with the concomitant accumulation of acid equivalents in the form of hexosemonophosphate and hexosediphosphate without the equivalent formation of lactic acid. The subsequent addition of phosphate directly or by its regeneration by adenosine triphosphatase or by its substitute, arsenate, permitted these hexose esters to metabolize further to produce lactic acid.

The situation with glucose-6-phosphate is somewhat different. In this case despite the presence of excess inorganic phosphate the evolution of CO_2 is more than twice as much as the formation of lactic acid. With the addition of adenosine diphosphate the increased formation of lactic acid now exceeds the amount of CO_2 evolved, the degree of excess depends upon the concentration of adenosine diphosphate added. With larger amounts of adenosine diphosphate the CO_2 evolution actually decreases. This was especially true in the presence of the high concentrations of adenosine monophosphate (twice that of adenosine diphosphate). Here both the lactic acid formation and CO_2 evolution rose rapidly in the first 10 minutes. As the reaction progressed, however, the lactic acid continued to be formed (fig. 3) while the evolution of CO_2 actually diminished to zero and below.

In the absence of added phosphate acceptor, adenosine diphosphate and adenosine monophosphate, it is presumed that further phosphorylated products of glucose-6-phosphate (probably fructose diphosphate and phosphoglyceric acid) begin to accumulate as acid equivalents and generate CO_2. In the presence of added adenosine diphosphate or adenosine monophosphate these acid equivalents do not accumulate but the inorganic phosphate is taken up by the adenosine diphosphate or adenosine monophosphate to give adenosine triphosphate or adenosine diphosphate, respectively. Since, according to the Embden-Meyerhof scheme for every mole of lactic acid two moles of adenosine triphosphate are formed,* this net uptake of inorganic phosphate results in a decrease of acid equivalents that is not compensated by the formation of lactic acid, or by the subsequent hydrolysis of adenosine triphosphate.

Discussion

In the normal course of the anaerobic metabolism of glucose by various tissues, the delicately balanced enzymatic integrity of the intact cells prevents the accumulation of intermediary products, since these are readily transformed into the end product, lactic acid. For a closer knowledge of the component reactions as well as the nature of the enzymes involved in a complex series of metabolic events, it is necessary to disrupt the cellular organization by experimental means so that the normal co-ordination of the vital processes is interrupted. Thus, the preparation of a cell-free extract is one means of studying the intermediary metabolism of the lens.

The demonstration of the dependence of the rate of glycolysis upon the presence of inorganic phosphate indicated the relative deficit of the enzyme adenosine triphos-

* This relationship was established for lens extract in the following paper.

phatase which controls regeneration of inorganic phosphate according to the reaction,

$$ATP \xrightarrow{ATP'ase} ADP + H_3PO_4$$

Indeed, the exhaustion of inorganic phosphate with the resulting slowing down of glycolysis could be counteracted by fortifying the lens extract with myosin adenosine triphosphatase. In the presence of the latter there was established a steady rate of glycolysis with the formation of lactic acid equal to that produced by the system fortified with inorganic phosphate. Enzymatic assay of the extract for adenosine triphosphatase showed no measurable activity, although Zeller et al.,[18] reported in rabbit lens homogenate the presence of adenosine triphosphatase capable of splitting 23.4 μM PO_4/hr./g. fresh tissue. Our results also indicate that the splitting of adenosine triphosphate by any nonspecific phosphatase is negligible.

Comparison of the rates of lactic acid formation from glucose, glucose-6- and fructose-6-phosphate, and glucose plus hexokinase indicates that the glucose-6-phosphate is probably the first product of glucose metabolism and that the concentration of hexokinase in the lens preparation is the rate limiting factor. The accelerating effect of added adenosine diphosphate and adenosine monophosphate again underscores the relative deficit of adenosine triphosphatase activity which would give rise to the formation of adenosine diphosphate. Of course, in the presence of added hexokinase, the transphosphorylation from adenosine triphosphate to glucose generates adenosine diphosphate fast enough to provide sufficient phosphate acceptor to make additional adenosine diphosphate unnecessary. Under these conditions the rate of lactic acid formation from glucose plus hexokinase is equal to that from glucose-6- or fructose-6-phosphate and adenosine monophosphate.

The identical behavior of glucose-6-phosphate and fructose-6-phosphate suggests a rapid interconversion mediated by the enzyme phosphohexoisomerase. It is also interesting to note that in a single experiment glucose-1-phosphate produced more lactic acid than did glucose. Whether glucose-1-phosphate or glucose-6-phosphate is the primary product of the phosphorylation of glucose remains to be established. In any event it would appear that the lens extract contains the enzyme phosphoglucomutase mediating the interconversion of these two phosphate esters.

Summary

Cell-free extracts of lenses were prepared from the eyes of rabbits 20 to 30 days of age and weighing between 0.75 and 1.0 lb. Their ability to form lactic acid under anaerobic conditions was studied in a Warburg apparatus at 37°C. In the absence of added inorganic phosphate the formation of lactic acid from glucose slowed down after about 40 minutes. The initial rate could be prolonged by fortifying the system with inorganic phosphate, either before or after the reaction had slowed down, or with arsenate or adenosine triphosphatase added after 60 minutes of reaction.

Increased amounts of lactic acid were formed when glucose-6-phosphate, fructose-6-phosphate, or glucose-1-phosphate were substituted for glucose. The addition of hexokinase very markedly increased the formation of lactic acid, considerably exceeding that produced by the hexosephosphates. By supplementing the latter with adenosine diphosphate or adenosine monophosphate the rate and extent of lactic acid produced was equal to that from glucose plus hexokinase. The limiting factors in the rate and extent of glycolysis by lens extract were shown to be inorganic phosphate, adenosine triphosphatase and hexokinase. The lack of correlation between the evolution of CO_2 from bicarbonate and the formation of lactic acid was discussed.

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ANAEROBIC CARBOHYDRATE METABOLISM OF THE CRYSTALLINE LENS*

II. Fructose diphosphate

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In Part I[1] of this series evidence was presented to show that the relative deficits of hexokinase and adenosine triphosphatase in lens extract were limiting factors in the production of lactic acid from glucose and glucose-6- and fructose-6-phosphate. When the glucose system was fortified with hexokinase and inorganic phosphate and the hexosemonophosphate system fortified with phosphate acceptor, adenosine diphosphate or adenosine monophosphate, increased quantities of lactic acid were formed. This report describes the anaerobic metabolism of fructose diphosphate by lens extract.

The preparation of the lens extract has already been described. The materials, procedures, and experimental techniques were the same as previously described. In addition the barium salt of fructose diphosphate (Schwarz laboratories), 98-percent pure by analysis with zymohexase,[2] was used.

* From the Research Department, Wills Eye Hospital. This work was supported in part by a grant-in-aid from the National Council to Combat Blindness.

Results

When fructose diphosphate was incubated with lens extract, with or without added inorganic phosphate, the evolution of CO_2 was considerably faster and greater than the corresponding formation of lactic acid (fig. 1). The addition of 1.0 mg. diphosphopyridine nucleotide had no measurable effect upon either process. The addition of adenosine triphosphatase, on the other hand, increased the initial rate and overall extent of evolution of CO_2, and, after an initial slow period of 30 minutes, markedly increased the initial rate and overall formation of lactic acid to equal that of the evolution of CO_2. Indeed, after 60 minutes it is apparent from Figure 1 that the rate of lactic-acid formation is faster than the rate of CO_2 evolution so that over a longer period of time the lactic acid formation may even exceed the CO_2 evolution.*

* In a well-balanced enzymic system the metabolism of fructose diphosphate to lactic acid results in a measurable increase in the pH of the reaction mixture.[5] This causes a retention of some CO_2 and is reflected in the quantity of lactic acid developed exceeding the amount of CO_2 liberated.

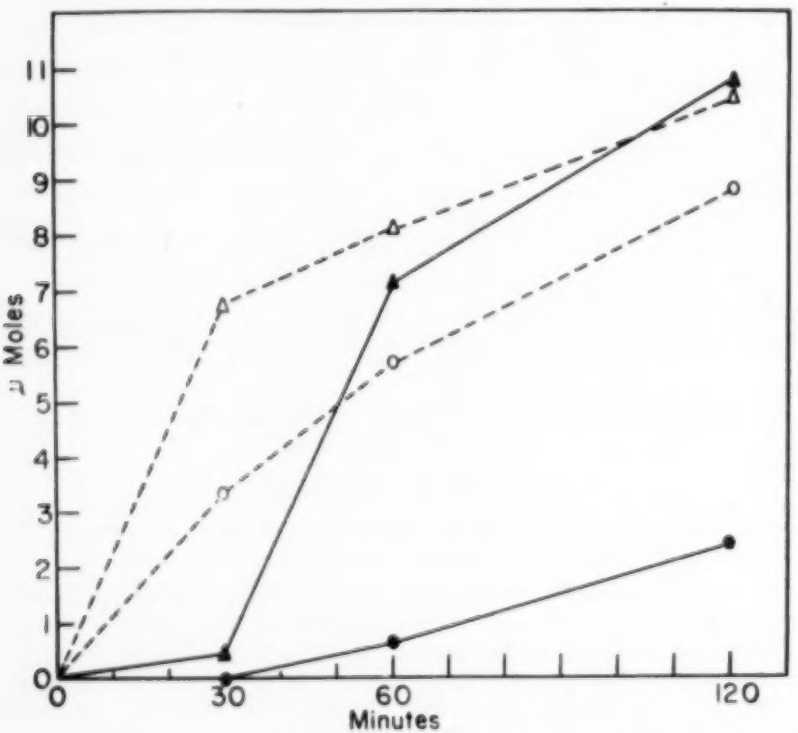

Fig. 1 (Green, Bocher, and Leopold). *Metabolism of fructose diphosphate in the presence of added adenosine triphosphatase.*

Main compartment: 0.4 ml. adenosine triphosphatase, 0.5 ml. isotonic KCl-$NaHCO_3$. Side-arm 1: 0.1 ml. fructose diphosphate (final concentration, 6×10^{-4}M); 0.05 ml. $CaCl_2$ (final concentration, 1.06×10^{-4}M); 0.05 ml. 0.1M PO_4 (final concentration, 2.94×10^{-4}M); 0.05 ml. 0.031M $NaHCO_3$; 0.05 ml. H_2O. Side-arm 2: 0.5 ml. extract. T.V., 1.7 ml.

Side arm contents tipped in at 0 minutes.

—○—○—, CO_2 production; —●—●—, lactic acid production, no adenosine triphosphatase.

—△—△—, CO_2 production; —▲—▲—, lactic acid production, with adenosine triphosphatase.

That the formation of lactic acid from fructose diphosphate by lens extract depended essentially upon the presence of sufficient amounts of suitable phosphate acceptor is supported by the results in Table 1. It is clear that both adenosine monophosphate and adenosine diphosphate caused a marked increase in the production of lactic acid. The addition of 6.67×10^{-3}M of creatine had no effect either upon the CO_2 evolution or lactic acid formation. Also the presence of 1×10^{-4}M dinitrophenol was without effect.

According to the classical Embden-Meyerhof scheme of anaerobic carbohydrate metabolism, when one mole of fructose diphosphate undergoes anaerobic metabolism, two moles of lactic acid are formed accompanied by the generation of four moles of adenosine triphosphate or 7′P and the disappearance of two moles of inorganic phosphate. This correlation for lens metabolism is given in Table 1 under Ratio.

The value of lactic acid was taken as one and compared with the values of the inorganic phosphate disappeared and the 7′P generated. In the first 30 minutes the disappearance of inorganic phosphate and the generation of 7′P were larger in proportion to the formation of lactic acid. After 60 minutes the ratio between lactic acid and 7′P was 1:2.2 with adenosine monophosphate and 1:1.9 with adenosine diphosphate. The inorganic phosphate disappearance however, was still relatively high.

After 120 minutes, in the case of adenosine monophosphate, the ratios of 1:1.9:1.3, respectively, for lactic acid, 7′P, and inorganic phosphate were obtained in good agreement with the Embden-Meyerhof scheme. In the case of adenosine diphosphate similar ratios were obtained, although the value given for 7′P may be low.

Attempts were made to determine the extent of disappearance of fructose diphosphate by the zymohexase method. Because of the relatively high concentration of inorganic phosphate and the small amount of fructose diphosphate remaining, the analyses were inaccurate. The correlation between the disappearance of fructose diphosphate and the formation of lactic acid was deter-

TABLE 1

CORRELATION OF THE FORMATION OF LACTIC ACID WITH THE GENERATION OF 7′P AND THE DISAPPEARANCE OF INORGANIC PHOSPHATE DURING THE METABOLISM OF FRUCTOSE DIPHOSPHATE

Additions	Time	Inorg. P.	7′P*	Lactic Acid	Ratio		
	Mins.	μM	μM	μM	Inorg. p	7′P	Lactic Acid
—	0	(48.10)	(7.74)	(5.43)	—	—	—
	60	3.93	−1.96	0.31	—	—	—
	120	3.29	−2.62	1.64	—	—	—
AMP	0	(48.30)	(7.70)	(6.78)	—	—	—
	30	9.60	16.40	5.75	1.7	2.9	1.0
	60	13.80	18.90	8.60	1.6	2.2	1.0
	120	16.50	23.30	12.40	1.3	1.9	1.0
ADP	0	(47.50)	(30.0)	(5.23)	—	—	—
	30	9.70	12.8	4.78	2.0	2.7	1.0
	60	11.00	11.5	6.20	1.8	1.9	1.0
	120	10.70	12.2	8.80	1.2	1.5	1.0

Main compartment: 0.5 ml. extract; 0.5 ml. isotonic KCl-$NaHCO_3$; 0.1 ml. H_2O.
Side-arm: 0.1 ml. FDP (final concentration, 3.33×10^{-3}M); 0.15 ml. 0.1 M PO_4 (final concentration, 1.33×10^{-3}M); 0.1 ml. ADP (final concentration, 6.67×10^{-3}M); 0.1 ml. AMP (final concentration, 1.33×10^{-3}M); 0.05 ml. 0.031 N $NaHCO_3$. Side-arm contents tipped in at 0 min. T.V., 1.5 ml.
* These values were not corrected for the quantity of 7′P disappearing with the metabolism of fructose diphosphate, 30 percent of whose organic phosphate is hydrolyzed in seven minutes.

TABLE 2

METABOLISM OF FRUCTOSE DIPHOSPHATE IN THE PRESENCE OF ARSENATE. CORRELATION OF LACTIC ACID FORMATION WITH THE DISAPPEARANCE OF FRUCTOSE DIPHOSPHATE, EVOLUTION OF CO_2 AND INCREASE IN INORGANIC PHOSPHATE

Time	CO_2	FDP-P	Inorg. P	Lactic Acid
Mins.	μM	μM	μM	μM
30	18.0	23.1	19.4	17.3
60	21.6	22.8	25.5	24.0
90	22.7	22.6	27.0	24.0

Main compartment: 0.5 ml. extract; 0.5 ml. isotonic KCL-$NaHCO_3$; 0.2 ml. H_2O.
Side-arm: 0.1 ml. FDP (final concentration, 3.1×10^{-2}M); 0.15 ml. A_8O_8 (final concentration, 2.08×10^{-2}M); 0.05 ml. 0.031 N $NaHCO_3$. Side-arm contents tipped in at 0 min. T.V., 1.5 ml.

mined in the absence of added inorganic phosphate but in the presence of A_8O_4.

The results in Table 2 show clearly the excellent agreement between the lactic acid developed, the CO_2 evolved,* the fructose diphosphate disappeared, and the inorganic phosphate formed. With regard to the latter it must be remembered that in the presence of arsenate, the phosphate of fructose diphosphate ends up finally as inorganic phosphate, rather than as 7′P.[6]

DISCUSSION

In evaluating whether a presumed substrate lies on the main pathway of glucose metabolism by lens extract, it is apparent, from this study, that unless the enzyme system is supplemented with proper cofactors, incorrect conclusions may be drawn. Thus the relatively slight ability of fructose diphosphate to give rise to lactic acid may be construed as indicating that fructose diphosphate is not an intermediary in lens metabolism. On the contrary, the high rate of lactic-acid formation in the presence of suitable phosphate acceptor demonstrated that fructose diphosphate is quite likely a definite metabolite in the utilization of sugar by the lens. This dependence upon the presence of adenosine diphosphate or adenosine monophosphate together with the demonstration that in the presence of A_8O_4 the production of lactic acid was completely dissociated from the uptake of inorganic phosphate pointed to the relative deficit of adenosinetriphosphatase activity in lens extract, confirming the findings in Part I of this series.

This study has shown that a stoichiometric relationship exists between the metabolism of fructose diphosphate and the production of lactic acid, the generation of high energy phosphate bonds and the uptake of inorganic phosphate. Although the presence of labile phosphate or adenosine diphosphate and adenosine triphosphate has been demonstrated[7, 8] in the crystalline lens and the uptake of P^{32} by intact lenses led to an increase in labile phosphorus,[9, 10] this is the first demonstration that the metabolic activity of the crystalline lens gives rise to the generation of high energy phosphate bonds and that these two processes are quantitatively related.

The results so far obtained are in accord with the glycolytic scheme of Embden and Meyerhof and suggest that the anaerobic utilization of glucose by lens extract follows a pathway essentially similar to that described for yeast, muscle, and other mammalian tissues.

Nordmann and Mandel[7] and Frohman and Kinsey[8] have reported the presence of creatin phosphate in animal lenses, and the latter authors suggest that this is the form in which the high energy phosphate of

* The average ratio of lactic acid formed to CO_2 evolved in the metabolism of fructose diphosphate in the presence of A_8O_4 was 1.3, in 12 experiments and for different time periods. Meyerhof[6] obtained a value of 1.24 with brewers' yeast.

adenosine triphosphate may be stored in the intact lens. In our experiments with lens extract creatin had no effect upon the lactic acid formation in the metabolism of fructose diphosphate and, therefore, could not act as a phosphate acceptor under these conditions. It would appear, therefore, that creatin phosphorylase, the enzyme that mediates the transfer of phosphate from adenosine triphosphate to creatin, was absent in our lens extract.

The relatively large evolution of CO_2 compared with the formation of lactic acid that occurred in the metabolism of fructose diphosphate in the absence of phosphate acceptor was probably due to the accumulation of an acid other than lactic. The probability that this may be phosphoglyceric acid is being investigated.

In the experiment with added adenosine triphosphatase the initial lag in the production of lactic acid during the first 30 minutes can be ascribed to the time necessary to generate sufficient phosphate acceptor, adenosine diphosphate.

Summary

The anaerobic metabolism of fructose diphosphate by an extract of rabbit lens was studied. Fructose diphosphate, with or without added inorganic phosphate, formed very little lactic acid. In the presence of a suitable phosphate acceptor, adenosine diphosphate or adenosine monophosphate, considerable quantities of lactic acid were produced.

Similar results were obtained in the presence of muscle adenosine triphosphatase or arsenate. When fructose diphosphate was fortified with adenosine di- or monophosphate, the production of one mole of lactic acid was accompanied by the generation of two moles of seven-minute phosphorus and the uptake of one mole of inorganic phosphate. In the presence of arsenate a rapid production of lactic acid occurred and a stoichiometric relationship was shown to exist between the fructose diphosphate metabolized and the lactic acid produced, the inorganic phosphate liberated and the CO_2 evolved.

The results are in accord with the glycolytic scheme of Embden and Meyerhof and suggest that the anaerobic utilization of glucose by lens extract follow a pathway essentially similar to that described for yeast, muscle, and other mammalian tissues.

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Discussion

Dr. John Harris: In some of our work on cation imbalance we have been able to demonstrate that adenotriphosphate in particular seems to have quite a toxic influence so far as our measure of tissue viability is concerned. In other words, in the presence of even quite low concentrations of adenotriphosphate we found a depression of the cation concentrating mechanism.

One of the things we observed to negate this depression—that is, to overcome the inhibition of the cation concentrating mechanism—was a relatively high glucose level. I wonder whether Dr. Dische has anything that would help us out in that respect, whether he has observed any quantitative relationship between the utilization of glucose and various concentrations of adenotriphosphate.

I would like to ask Dr. Green whether he has tried insulin in some of his lenticular homogenates. You will recall that in its presence Ross, in England, was able to demonstrate a greater uptake of glucose from the media in the decapsulated but not necessarily homogenized tissue.

Dr. Zacharias Dische: Dr. Harris asked about the correlation between adenotriphosphate concentration and glucose. I can only say that we also tried much higher concentrations of adenotriphosphate—10 times as much as there were here. There was no influence whatever. In very much higher concentrations the effect was almost the same as in the low concentrations.

On the other hand, the degree to which adenotriphosphate preparation was broken down was irregular and erratic—apparently it depends upon the care with which the capsule is stripped off. There is probably a very great increase of the adenotriphosphate activity when the capsule is stripped off, because there are certain structural changes in the capsule which activate either the transference relations or the adenotriphosphate.

It would seem that adenotriphosphate would rather depress the active cation transport. It may be due to some specific effect of adenotriphosphate on the proteins, which can be expected to a very high degree.

I am very much impressed by the elegance of the experiments of Dr. Green and this equivalence of phosphate, lactic acid, carbon dioxide, and adenotriphosphate.

I would like to say I did not understand whether this was done on the whole lens or on homogenates.

Also, I would like to ask Dr. Green how he explains that in his experiments the glucose-6-phosphate had a higher rate of turnover than glucose itself, although the amount of the phosphate acceptor is the limiting factor, and particularly as monophosphate is apparently a better acceptor in his experiments than is adenotriphosphate.

Under these circumstances we would expect that glucose would create more acceptors than glucose-6-phosphate, and for this reason the rate of the turnover with glucose would be higher than glucose-6-phosphate, because monophosphate appears as a better aceptor. That is rather unusual.

I would not think this would be the case in general. I would think adenodiphosphate would be the better acceptor than the adenotriphosphate. In his experiments, adenodiphosphate was better than adenotriphosphate. Without a complete analysis of the balance, of how much adenodiphosphate was used, I wonder how you can reconcile these findings with the fact that the amount of the phosphate acceptors is the limiting factor in the experiments.

Dr. Harry Green: Thank you, Dr. Dische, for your fine remarks. We used cell-free extracts of the entire lens, capsule and all. They were adjusted to a pH of 7.4.

As to the difference between glucose and glucose-6-phosphate, the limiting factor is not the phosphate acceptor but the relative deficit of hexokinase in the lens extracts, so that in the presence of added hexokinase glucose will produce lactic acid considerably faster and to a greater extent than that produced by glucose-6-phosphate.

In this case, in the presence of hexokinase, there occurs a very rapid phosphorylation of glucose, which produces a considerably larger amount of phosphate acceptor in a relatively short period of time. The result is a higher concentration of phosphate acceptor than originally present in lens extract.

If the phosphate acceptor is added to the glucose-6-phosphate system, the two rates become equal. The limiting factor is, therefore, not the adenosine diphosphate, but the hexokinase. Once hexokinase is present, however, then the limiting factor is the phosphate acceptor.

It is true that we have found that adenosine monophosphate is a relatively more efficient phosphate acceptor than the adenosine diphosphate. I don't agree that this observation is rather unusual; but, be that as it may, it would suggest to us that the phosphorylase enzyme responsible for the uptake of phosphorus by adenosine diphosphate is perhaps less active than that responsible for the phosphorylation of adenosine monophosphate. That remains to be established.

With regard to the inhibitory properties of adenosine triphosphate observed by Dr. Harris, we have found that an excess of adenosine triphosphate inhibited glycolysis in the presence of added hexokinase.

AN EVALUATION OF INTRAOCULAR STREPTOKINASE*

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The identification of the fibrinolytic enzyme activator, streptokinase, was an ougrowth of research efforts in the field of bacterial exotoxins. Early in their investigations of hemolytic streptococci, Tillet and his coworkers[30, 35] observed that broth cultures of the organisms could cause rapid lysis in fibrin clots of human blood. This lytic property was found to be extracellular and to be secreted into the culture medium during growth, by the organism.[35] Isolation and assay of the principle soon followed and the name streptokinase was assigned by Christensen.[6]

Among the animal blood types tested in early experiments, only clots of human fibrin appeared susceptible to liquefaction by the streptococcal principle. Thus it was that studies were undertaken to identify a human serum factor necessary to the streptokinase fibrinolysis reaction, the absence of which precluded any such reaction.

The existence of such a factor was confirmed by Milstone[20] who detected its presence in the euglobulin fraction of human plasma. It is interesting to note that the existence of his euglobulin factor in human blood had been demonstrated some years earlier and its fibrinolytic function discussed.

Dastre,[11] for example, noted dissolution of human clots immersed in human serum for 18 hours: this was in 1893. In 1903, the fibrinolytic activity of human serum was shown to be increased in addition of chloroform.[12] The following year, Hedin, and others, showed this autofibrinolytic activity of human blood was a function of the euglobulin fraction of the serum.[15-19]

More recent attempts to assay this euglobulin factor have confirmed its enzymatic nature. The inactive form has been named plasminogen by Christensen. When activated the enzyme is called plasmin. The following reaction sequence involving the activator substance, streptokinase and the serum enzyme, plasminogen, has been postulated:[6]

1. Plasminogen is the inactive form of an enzyme capable of causing fibrinolysis.

2. Streptokinase changes plasminogen rapidly and completely to an active form plasmin.

3. Plasmin then catalyzes the fibrinolysis.

Subsequent studies of the chemical nature and purity of streptokinase demonstrated the presence of a desoxyribonuclease (called streptodornase, by Christensen[37]), hyaluronidase, and antienzyme factors in significant quantities.[21] It is not available as a purified preparation. Effects of its impurities are greater in high concentrations of streptokinase.[6]

Although many other physical, chemical and pharmacologic agents have exhibited similar properties,[2, 3, 5, 12, 24, 27, 28] none possesses the efficiency of streptokinase in this regard.

Selye[24] asserts that activation of a fibrinolytic blood principle by so wide a variety of stimuli shows the activation to be a manifestation of stress response in the human organism. The striking parallelism between the severity of many rheumatic-type diseases and the antifibrinolytic activity of the sera is sighted by many to support this position.[29, 33, 34]

It is felt that the antigenic response to streptokinase is part of this same picture.[8]

Also of significance in this regard is the increase of the antiphlasmin activity of hu-

* From the Department of Research of the Wills Eye Hospital. Thesis submitted to the faculty of the Graduate School of Medicine of the University of Pennsylvania toward the requirements for the Degree of Master of Medical Science (M.Sc. (Med.)) for graduate work in ophthalmology.

man serum after administration of ATCH or cortisone.[24]

The recognition of a plasmin fibrinolytic factor and anti-enzyme plasmin inhibitors as balancing components of the blood proteolytic system greatly facilitates an approach to the problem of fibrinolysis in vivo.[43] Although the nature of such antienzyme factors is as yet unknown their presence is certain.[6, 14, 17, 22, 29]

Streptokinase has been employed to aid in lysis and removal of fibrin compounded exudate from many of the body areas.[25, 26, 32, 36, 38-40] Treatment method and periodicity have varied with the size and location of the site considered. The need for surgical drainage has not been obviated, however, and aspiration is often required in dealing with closed or semiclosed cavities.

Because the presence of suitable substrate in the form of clotted blood is seen clinically in patients with complicated hyphema, the role of intraocular streptokinase has come under consideration. The manner of action of the agent and the contemplated locus of employ make the following factors seem pertinent:

1. The anterior chamber represents a semiclosed space constructed of highly specialized and sensitive tissues. It is contantly perfused by aqueous humor which will serve to dilute and to remove by flow or diffusion many water soluble agents introduced.

2. The degree of fibrinolytic activity in a given area is proportional to the concentration of activated serum plasmin and not to the streptokinase concentration. An excess of the latter component assures only maximal rate of activation of available serum plasminogen.[8]

3. The time required to effect fibrinolysis of a clot mass is dependent upon the concentration of substrate fibrin and active plasmin.[5, 14, 22] The relationship of fibrinolysis time to the proportion of these two substances is as a straight line graph.[8]

4. Contact of substrate with streptokinase must obtain for sufficient time to permit activation of available plasminogen.[8] Factors of contact time and surface area thus become significant.

5. As presently available, streptokinase is not a pure preparation. The impurities present are more active in higher concentrations of the streptokinase preparation.[7]

Several of the foregoing limitations are unique to a consideration of the anterior chamber. Other body areas which permit of larger clots and easier access to serum elements would less likely present the possibility of incomplete fibrinolysis.

In an effort to study the relative importance of these factors the investigation of intraocular behavior of streptokinase was undertaken in the following sequence:

Section I. Procedures and Methods: Standardization of analyses methods.

Section II. Studies of ocular tolerance to streptokinase.

Section III. Duration of activity studies.

Section IV. Studies of anterior chamber antistreptokinase activity.

Section V. Studies of treatment of experimental lesions.

Section VI. Discussion and summary.

Section VII. Conclusions.

Section I. Procedures and Methods

It is the purpose of this section to present in adequate detail the methods of animal surgery and biochemical assay employed in this study. The sequence is as follows:

1. *Preliminary in vitro studies of whole blood clots*
   - A. Preparation of standard clots
   - B. Effect of clot composition on streptokinase fibrinolysis
   - C. Effect of temperature on fibrinolysis in vitro
2. *Description of preparations for animal injection*
   - A. Anesthesia: General, surface, and local
   - B. Preoperative and postoperative care

3. *Description of lesion production, treatment and aqueous recovery*
   A. Simple hyphema
   B. Secondary glaucoma
   C. Streptokinase and saline injections
   D. Simple paracentesis
4. *Biochemical assay methods*
   A. Drugs and solutions used
   B. Fibrinolysis time, streptokinase
   C. Fibrinolysis time, plasmin
   D. Clot inhibition test
   E. Antistreptokinase testing method

I. *Preliminary in vitro studies of whole blood clots*

The following studies were done to aid in the selection of a proper substrate for use in lesion production. Previous investigations have indicated that fibrinolysis proceeds more rapidly in human blood clots than in those of animal blood.[19, 20]

A. Preparation of standard clots

Five cc. of whole blood was placed in a small test tube of 1.5 cm. and 1.0 cc. of bovine thrombin in concentration of 200 units per cc. was added. Prompt and repeated inversion of the tube assured thorough mixture of contents prior to clotting. The tube is then placed in an ice water bath for five minutes to allow firm clotting. The clot, when removed by loosening the edges gently with a wire loop, forms a cast of the tube bore. This was placed on a clean dry glass surface and promptly cut with iris scissors into wafers of 0.5 cm. thickness. With the aid of a glass rod the clots were directed into the individual test tubes in the water bath as required.

Fresh clots were prepared for each series of tests and constant sources of human blood, human plasma, and of rabbit blood were used throughout this section of the study. In this way factors of surface area and of plasminogen content remained uniform.

In this manner three clot types were prepared using human blood, rabbit blood, and rabbit blood mixed with an equal volume of human plasma.

B. Effect of clot composition on streptokinase fibrinolysis

Each of the three clot types was then tested as follows:

Fifteen Kahn-type test tubes containing each 0.1 cc. of freshly prepared streptokinase solution were immersed in the water bath to a depth of two inches. Five different dilutions in range of 5,000 units per cc. to 50,000 units per cc. were employed and a temperature of 37°C. was constant in the water bath. Three control tubes each containing 0.1 cc. of normal saline were immersed simultaneously.

Each of the three clot types described was incubated with five different dilutions of streptokinase solution. Dissolution was taken as completed when tube contents flowed freely and introduction of a glass rod showed no solid matter remaining. The tubes were flooded with water when the presence of any particulate matter was suspected.

Results shown in Table 1, indicate that clots of rabbit blood and those of mixed composition are poorly lysed. Whole human blood proved the most susceptible of the substrate materials tested and was used in further vivo tests.

C. Effect of temperature on fibrinolysis in vitro

As with many enzyme factors, so with streptokinase, an increase in temperature is held to increase enzymatic activity within specific limits of range. Preliminary studies on temperature effect were done in a manner similar to that described above in Section B.

Freshly prepared human clots were used in each of four series conducted at temperatures of 37°, 40°, 43°, and 45°C., respectively. Eighteen test tubes, three as controls

TABLE I

PRELIMINARY STUDY: RELATIVE SUSCEPTIBILITY OF CLOTS OF HUMAN AND RABBIT BLOOD TO FIBRINOLYSIS

Streptokinase Concentration	Rabbit Blood	Human Blood	Rabbit Blood and Human Serum
Units/cc.	Time Required for Lysis of Clots		
	Hours	Minute	Hours
50,000	14	55	4
25,000	23	78	5
15,000	16	89	8½
10,000	29	187	6
5,000	Neg. at 48	8 hr.	14
Control	Negative at 48 hr.		

and 15 containing streptokinase in concentration range from 5,000 units per cc. to 50,000 units per cc., comprised each series.

Results, as shown in Chart 1, indicated a definite reduction in time of clot dissolution coincident with an increase of incubation temperature. Inactivation of streptokinase has been shown to occur above a temperature of 50°C. Body temperature elevation to this range is not feasible. Subsequent attempts at induction of fever in rabbits were not successful in our hands.

2. *Description of preparations for animal injection*

A. ANESTHESIA: GENERAL, SURFACE, AND LOCAL

General anesthesia was reserved for those methods of lesion induction and treatment where accuracy demanded complete animal control. This was especially true of those procedures of anterior chamber injection wherein loss by regurgitation had to be minimized. The anesthesia employed was Pentobarbital sodium containing 60 mg. of barbiturate per cc. This was diluted to a volume of at least 1.0 cc. and injected slowly via the marginal ear vein, during a period of no less than two minutes. When dilution to attain the 1.0 cc. volume minimum was required, sterile normal saline solution was added.

Rapid injection or use of too concentrated a solution resulted in anesthetic deaths, especially in animals of body weight below 1,500 gm. Adult rabbits in weight range of 1,200 to 2,000 gm. were used in subsequent series. Following induction of anesthesia animals were placed in rabbit boxes with heads erect. Periodic irrigation of exposed corneas was maintained.

Local anesthesia was reserved for such procedures as paracentesis wherein complete control was not as difficult to maintain. After irrigation with 1:10,000 Metaphen solution, a sterile solution of one-percent tetracaine hydrochloride was instilled into each conjunctival sac. When aided by good

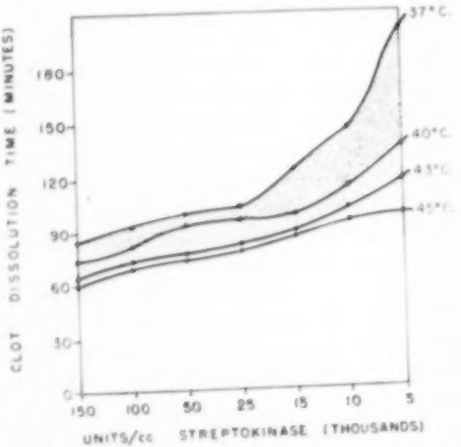

Chart 1 (O'Rourke). Effect of temperature and of streptokinase concentration on in vitro dissolution of prepared clots.

manual control of the animal head, this method proved adequate for such procedures.

When an animal proved unmanageable with surface anesthesia, a retrobulbar injection of 0.5 cc. of a 2.0-percent procaine solution was given. Injections were performed with a 21-gauge needle which was inserted into the retrobulbar space through the inferior nasal cul-de-sac. Ready access to this area was afforded by preliminary proptosis of the globe. This was effected by pressure with the angled edge of a muscle hook, beneath the globe.

B. Preoperative and postoperative care

1. Control of septic factors was obtained by sterilizing all instruments, syringes, and needles in boiling water for 30 minutes. These were then immersed in a solution of Zephiran chloride immediately before use.

2. All rabbit eyes consigned for intraocular procedures received a thorough irrigation with a 1:10,000 Metaphen solution. This was repeated in 10 minutes. The second flush was followed in 10 minutes by irrigation of these areas with sterile saline. In cases of general anesthesia where corneal exposure was a factor periodic irrigation with sterile saline was done.

3. After any procedure done under general anesthesia, a median tarsorrhaphy was performed and 1:10,000 Metaphen ointment was instilled into the lower cul-de-sac. Animal recovery was then allowed to proceed in animal boxes designed to hold heads erect. When recovery was completed sutures were removed and complete irrigation with 1:10,000 Metaphen solution and saline was done. With the above methods significant infection loss of rabbit eyes was held to less than two percent of 300 rabbits used.

3. *Description of lesion production, treatment, and aqueous recovery*

A. Simple hyphema

Well-anesthetized, adult rabbits were placed on sterile towels until needed. Both eyes were flushed and irrigated. When ready for injection, they were placed on a board covered with a sterile towel and the lids retracted manually by a gloved assistant.

Human blood was drawn into a sterile 10-cc. syringe from the antecubital vein of a constant donor and at once placed in a sterile dry test tube. Without undue delay, this was drawn into dry, sterile tuberculin syringes, to the level of 0.25 cc., and handed to the operator by an assistant.

A puncture of the perilimbal bulbar conjunctiva, beginning about three mm. from the nasal limbus, was made with a 26-gauge needle on a sterile, dry tuberculin syringe. The needle was directed to the limbal area at which point the anterior chamber was entered. A paracentesis was done slowly and yielded from 0.2 cc. to 0.25 cc. of clear aqueous.

Promptly, a syringe containing human blood was substituted at the adapter end of the needle and 0.2 cc. injected into the anterior chamber. The needle was gently withdrawn as the puncture was covered with a sterile cotton sponge soaked in bovine thrombin solution in concentration of 500 units per cc.

With well-anesthetized animals, it was found possible to inject between 16 and 20 eyes before coagulation of the blood specimen occurred. When human plasma was injected a similar technique was employed in later series.

B. Secondary glaucoma

This lesion type was induced by the injection of 0.1 cc. of whole human blood, after which a syringe containing 0.5 cc. of bovine thrombin solution was substituted and the contents barbotaged three times to achieve mixture prior to coagulation. A thrombin concentration of 500 units per cc. of solution was used. Coagulation of injected matter was prompt and resulted in many glaucomatous eyes. Spontaneous rupture of these globes occurred frequently after 48 hours. Only the most gentle handling of

such eyes prevented rupture at the limbal area. For this reason new sharp 26-gauge needles of one-half inch length were favored for injection and aspiration procedures in this section.

C. Streptokinase and saline injection

Regurgitation loss was a significant danger in this section also. Because of this, general anesthesia was used. Ten adult rabbits were inducted for each series at one time. Best retention of noncoagulable solutions such as these was obtained when an oblique transcorneal puncture was used.

With lid retraction and forceps fixation at the superior rectus insertion, the puncture was begun two mm. inside the superior nasal limbus and continued laterally at an angle of about 30 degrees below the horizontal until the anterior chamber was entered. The aqueous was aspirated into a dry, sterile syringe which was then disengaged from the needle. A second sterile syringe containing 0.2 cc. of the solution for injection was inserted into the needle and the injection completed. As the needle was withdrawn the puncture site was covered with a thrombin soaked sponge, to offset regurgitation loss.

D. Simple paracentesis

Recovery of aqueous specimens by paracentesis for study of enzyme activity was accomplished under local anesthesia. A perilimbal puncture with a 26-gauge needle was employed with aseptic precaution as described.

4. *Biochemical assay methods*

Testing methods for presence of streptokinase activity are based on two of its properties.

a. Ability, at body temperature, to effect the rapid conversion of plasminogen to plasmin, as manifested by lysis of fibrin present.

b. Ability to act upon fibrinogen in such a way as to prevent its subsequent coagulation despite addition of excess thrombin.

A 0.25-percent solution of bovine fibrinogen was used as a constant testing substrate of uniform clottable protein content. Fresh solutions were prepared for each series of tests. This was done to avoid the precipitation caused by temperature changes on standing.

Tests performed on this fibrinogen substrate were in reality tests for the presence of plasmin. Thus when testing for free streptokinase, it was necessary to permit preliminary incubation of the sample with an aliquot of human plasma for 30 minutes. The presence of free streptokinase within body fluid could be detected best by a demonstration of its ability to activate human plasminogen. Then the activity of this activated plasma sample is tested on the bovine fibrinogen substrate.

This added step was necessary because direct mixture of streptokinase solution with bovine fibrinogen when followed by coagulation with thrombin did not result in effective fibrinolysis of the substrate. This is because of the previously described reduced effectiveness of streptokinase fibrinolysis on animal plasma, in the absence of human plasmin factor.[14,28] Constant sources of human plasma, fibrinogen, and streptokinase have maintained in all series done for comparison.

A. Drugs and solutions used

Streptokinase. This was supplied by Lederle Laboratories as Varidase in 30-cc. vials. Each contained in powdered form 100,000 units of streptokinase and 25,000 units of streptodornase. Unmeasured amounts of hyaluronidase, streptolysin O, streptolysin S, ribonuclease, and other antienzyme factors are present. The significance of these impurity types decreases in the higher dilutions of the powdered product. It was this last factor that prompted the use of higher concentrations during in vivo studies of antienzyme factors, and

injection of less toxic concentrations in treatment series.

The recommended storage temperature range of 2° to 10° was observed. The streptokinase powder contained 0.01-percent sodium ethyl mercury thiosulphate as a preservative plus an added buffer to maintain a pH of 7.5 The material goes into solution easily upon injection of the desired amount of sterile saline. Violent shaking is contraindicated. Solution was made fresh for each test series because the deterioration at room temperature can be significant. Any specimen aspirated from the anterior chamber was promptly assayed, for the same reason.

Bovine thrombin. This was obtained as sterile thrombin from the Upjohn Company in 30-cc. vials, each containing 1,000 units as dried powder. A unit is described as the amount needed to effect clotting on 0.1 cc. of a 1.0-percent fibrinogen solution in 15 seconds at 28°C. of temperature. As used in these studies the contents of each vial were dissolved in 2.0 cc. of sterile physiologic saline solution to give a concentration of 500 units per cc. Because of potency loss on standing at room temperature, thrombin solution was prepared anew for each test series.

Hyaluronidase. This material was obtained from Wyeth Chemical Company as a highly concentrated dried powder extract of bull testes containing 1,300 turbidity reducing units per mg. The purity of this material was greater than that of presently available commercial hylauronidase.

Bovine fibrinogen. This material, bovine fibrinogen, fraction 1 (Cohn nomenclature) was purchased from the research division of Armour and Company. This was received as dried powder which because of the extraction process contained considerable quantities of sodium citrate. A constant source of this powder was employed for all test series because the clottable protein can how up to 10-percent difference by weight in any two lots. The clottable protein of the sample used in these studies was found to be 48 percent by weight. Although this powder enters solution more readily in a vacuum flask, preparation of the 0.25-percent solution used here did not require this method.

An amount of 125 mg. of the powder was measured on the chain balance and added to 50 cc. of physiologic saline solution in a glass beaker. This was stirred with a glass rod until dissolved. The solution was also prepared fresh each day as precipitation is noted on chilling or standing.

Human plasma. To meet the need for human plasma of constant source and composition, several vials of Lyovac plasma were obtained from the Sharpe and Dohme Company, all of similar lot number. This assured similar content of activatable plasminogen in each aliquot used. Each vial contained the equivalent of 5.0 cc. of human plasma in dried form and was injected with 2.5 cc. of the diluent supplied to yield a liquid form of double the human strength.

Activated plasma as used in the duration of action series was prepared by the injection 2.5 cc. of 100,000 units per cc. concentration of streptokinase solution into the vail. Gentle swirling allowed dissolution of the plasma at 37°C. in the water bath. The vial was allowed to remain in the water bath for 30 minutes.

B. Fibrinolysis time-streptokinase

This procedure is designed to test the ability of aqueous humor to activate plasminogen.

1. Upon recovery by paracentesis, the aqueous humor specimen to be tested in 0.1 cc. amount was added to 0.2 of human plasma solution promptly. Incubation at 37°C. for 30 minutes in a water bath followed.

2. Then 0.1 cc. of this plasma solution was removed by pipette and added to a test tube placed in the water bath containing 0.8 cc. of a 0.25-percent bovine fibrinogen solu-

tion. These two solutions were well mixed by inverting the tube three times.

3. Next 0.2 cc. of a bovine thrombin solution in concentration of 200 units per cc. was added. The tube was again promptly inverted three times and returned to the bath. A firm opalescent clot formed at once, the dissolution of which was preceded by clearing of the opacity. Any failure of the clot to form promptly in this manner indicated a defect in one or all of the component solutions and was best avoided by routine preliminary check of their coagulability. The time of liquefaction was readily appreciated and timed by a stop watch.

Control tubes contained 0.1 cc. of normal saline solution in place of the aqueous humor specimen. When tested in the above manner an average fibrinolysis time for 20 separate specimens of streptokinase solution in concentration of 10,000 units per cc. was four minutes and 18 seconds with a range of three minutes, 26 seconds to four minutes, 52 seconds. In a concentration of 5,000 units per cc. the average of 20 tests was eight minutes, 18 seconds with a range from six minutes, 48 seconds to nine minutes, 24 seconds.

C. Fibrinolysis time-activated plasma (plasmin)

Preliminary incubation of the aqueous specimen was not necessary here as this is a test for presence of activated plasmin. Preparation of the plasma injected was described above.

1. The amount of 0.1 cc. of the aqueous humor to be tested for fibrinolytic activity was added to 0.8 cc. of 0.25-percent bovine fibrinogen solution in a test tube and the two were well mixed by inversion three times.

2. Bovine thrombin in amount of 0.2 cc. and of concentration 200 units per cc. was then added and the triple inversion repeated. A whitish clot similar to that described in the previous section formed and the end point of fibrinolysis was read in the same manner.

D. Clot inhibition test

This test was devised as a corollary to the fibrinolysis time and to test for the presence of free streptokinase. It is dependent on the action of free streptokinase on human fibrinogen which reduces the susceptibility of the latter to coagulation despite subsequent addition of excess thrombin. This is in accordance with results of Milstone.[20] Preliminary testing of serial dilutions of streptokinase solution showed that this property is lost below a concentration of 3,000 to 3,500 units per cc. Tests were done on 20 separate lots of streptokinase.

1. Human plasma solution, 0.8 cc., and 0.1 cc. of aqueous humor solution to be tested were incubated together in a test tube placed in the water bath at 37°C. The control tubes contained 0.8 cc. of plasma solution plus 0.1 cc. of normal saline solution.

2. Following this incubation, 0.2 cc. of bovine thrombin solution in 200 units per cc. concentration were added to each tube and thorough mixture assured by triple inversion. Failure of the contents to coagulate indicated presence of free streptokinase in concentration above 3,500 units per cc. Results were recorded as positive or negative.

E. Antistreptokinase testing method

This test showed inhibition of streptokinase activity by a demonstration of reduced plasminogen activating efficiency.

1. The amout of 0.1 cc. of aqueous solution to be tested was incubated with 0.1 cc. of streptokinase solution of concentration 20,000 units per cc. This continued for 60 minutes at a temperature of 37°C. Control tubes contained this same concentration of streptokinase plus 0.1 cc. saline.

2. Following this the total content of each tube was added to a tube containing 0.5 cc. of human plasma solution and the mixture

incubated at 37°C. for 30 minutes in all cases.

3. At the end of this period 0.1 cc. of the plasma mixture was drawn from each tube separately, and a fibrinolysis time test for presence of activated plasma performed, as described in Section C. End-point times were recorded as previously described. A marked difference in fibrinolysis time of test aqueous and control was presumed evidence of antistreptokinase action upon the fibrinolytic system present. Control tubes contained 0.1 cc. of normal saline plus 0.1 cc. of streptokinase solution of 20,000 units per cc. concentration.

Section II. Studies of ocular tolerance to streptokinase

Intraocular injection of streptokinase in high concentration has caused an acute inflammatory response of the anterior segment, according to Jukofsky.[16] This reaction was described as the prompt onset of corneal edema with varying degrees of anterior chamber cellular reaction. These reactions were best evaluated at the eight-hour interval and were less severe below 100,000 units per cc. Because the impurities contained in available streptokinase have not been isolated and estimated, it is difficult to evaluate their responsibility for this reaction. To study the ocular tolerance of this agent, animal eyes were injected with varying concentrations as follows:

Following paracentesis, 0.2 cc. of streptokinase solution was injected intracamerally into each of 24 rabbit eyes. Eight different concentrations were employed in range from 200,000 units to 5,000 units per cc. Each concentration was injected into six eyes. Previously described techniques and precautions were employed. Observations were made of the degree and duration of corneal edema, this being the most obvious of the toxic reactions seen. Selected eyes were sectioned for further study.

As shown in Table 2, corneal opacification was most pronounced above the concentration of 50,000 units per cc. This reaction appears as a uniform steaming of the stroma affecting the deeper layers first and clearing from the periphery in irregular fashion often leaving residual islets of opacity in the central areas for a few days. Biomicroscopic observation confirmed the appearance of edema and microscopic section showed the lamellar separation (fig. 1) associated with this. In general, the anterior chamber reaction had cleared by the time corneal clearing was accomplished. This was evidenced by absence of flare on biomicroscopic examination.

The relation of corneal clearing time to concentration was interesting and fairly constant. The clearing time of the 100,000 units per cc. dose was about one half that of the 200,000 units per cc. concentration. No corneal edema or anterior chamber reaction were noted following injections below concentration of 50,000 units per cc.

Many impurity factors, as mentioned, are known to be present and to be more active in higher concentration of streptokinase solution.

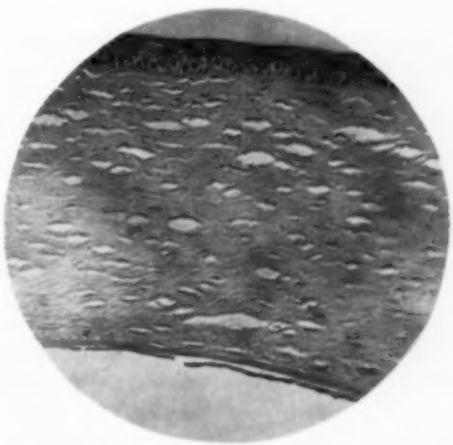

Fig. 1 (O'Rourke). Photomicrograph, showing corneal edema following intracameral injection of streptokinase in high concentration (250,000 units/cc.).

TABLE 2

REACTION OF OCULAR TISSUE FOLLOWING INJECTION OF STREPTOKINASE INTO ANTERIOR CHAMBER

Eye* No. ↓	Streptokinase Concentration (Units per cc.)									
	200,000		150,000		100,000		75,000	50,000	25,000	10,000	5,000
	Opaque Cornea							Clear Cornea			
	Opacity		Opacity		Opacity						
	%	Days	%	Days	%	Days					
I†	100	7	100	8	30	3	Cornea-opaque 30% 5 days				
II	100	8	100	6	50	4	Cornea-opaque 50% 5 days				
III	100	7	75	6	20 Aqueous cloudy	3 3	Cornea-clear Aqueous-cloudy 3 days				
IV	100	7	100	7	75	7	Cornea-clear Aqueous-clear				
V	100	6	75	7	60	5	Cornea-clear Aqueous-cloudy 5 days				
VI	100	8	100	6	60	4	Cornea-clear Aqueous-clear				
Average Clearing—Time (days)		7½		6⅔		4⅓					

* Each eye injected with 0.2 cc. of streptokinase solution.
† Six eyes per each concentration of streptokinase.

Hyaluronidase is the one impurity factor well identified and readily available for testing purposes. Intense inflammatory response followed injection of this agent.[18, 21, 42] Intraocular injections were done with hyaluronidase solution to compare the reaction pattern of the anterior segments.

A solution of hyaluronidase containing 750 turbidity units per cc. was injected in 0.2 cc. amounts into each of 10 rabbit eyes. Within two to four hours following this injection, a corneal edema pattern similar to that seen with streptokinase was observed (fig. 2).

The anterior chamber reaction of iritis appeared as more severe, longer lasting, and more prone to synechia formation than was streptokinase in highest concentration. The clearing time of the corneal edema exceeded

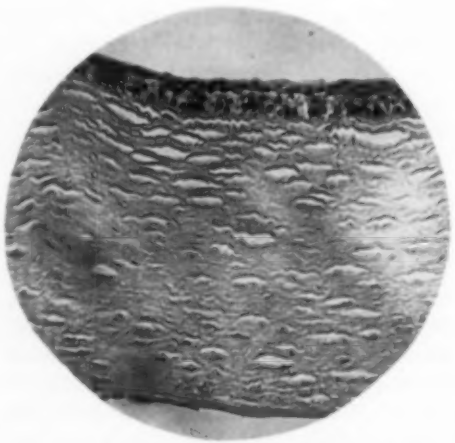

Fig. 2 (O'Rourke). Photomicrograph, showing corneal edema following intracameral injection of hyaluronidase in high concentration.

eight days in all eyes. The amount of streptococcal hyaluronidase present in any given concentration of streptokinase solution is not kown. A comparison of its activity to that of bull testis extracted hyaluronidase is also unknown.

Even with these limitations it is plausible to suspect that a great part of the anterior chamber and corneal reaction seen after injection of streptokinase of high concentration may be related to the contained hyaluronidase. Certainly the reaction pattern of endothelial damage is similar in each case. It is further known that use of high concentrations of streptokinase in no way increased the degree of intensity of the fibrinolytic process: This depends on the amount of activated plasminogen (plasmin) present at one time.[8, 14, 23]

Excess streptokinase assures only a maximal rate of activation of available plasminogen in the substrate and a suitable excess for clots of anterior chamber size can be found well below the toxic concentration range. The problem then becomes one of maintaining this suitable excess despite the aqueous humor outflow.

Section III. Duration of action studies

Preliminary in vitro studies of streptokinase fibrinolysis at body temperature have shown a time requirement of over one hour in all cases. Concentrations employed were within the limits of ocular tolerance. The in vivo fibrinolytic activity of streptokinase within the rabbit eye is subjected to three limiting factors which did not obtain during in vitro water bath studies. These factors are:

1. Constant dilution of injected streptokinase solution by inflow and egress of aqueous humor.

2. Paracentesis of anterior chamber prior to lesion induction (blood injection) encourages the inflow of a plasmoid aqueous rich in a fibrin type refractory to streptokinase action. Such fibrin by coating exposed clot surfaces could inhibit interaction with available streptokinase.

3. The possibility of a significant titer of antistreptokinase or antibody factors within the plasmoid rabbit aqueous. Their presence in animal sera have been mentioned by others.[8, 14, 17, 22, 29]

With such in mind a study of the duration of action within the anterior chamber was undertaken. Possible methods of prolonging it were also considered.

An aqueous solution of streptokinase in concentration of 50,000 units per cc. was injected into the anterior chamber of each of 48 adult rabbit eyes, following preliminary paracentesis. The average fibrinolysis time for 10 separate aliquots of this solution of streptokinase prior to injection was three minutes 38 seconds. The amount injected into each eye was 0.2 cc.

Paracenteses were performed at calculated intervals up to three and one-half hours. A fibrinolysis time determination was performed on 0.1 cc. of each specimen promptly, as previously described.

Clot inhibition studies were done also on the remaining portion (about 0.1 cc.) of each specimen of aqueous humor. A negative result indicated the absence of streptokinase above a concentration of 3,500 units per cc. and was used as a test for the presence of streptokinase above that level.

Chart 2 shows that, following the injection, significant fibrinolytic activity is lost well before one hour has elapsed. The fibrinolysis time of one hour's aqueous humor specimen was 20 to 25 times as long (64 to 86 minutes) as that of the solution prior to injection three minutes, 38 seconds. The function of clot inhibition was lost in 30 to 45 minutes. This represented a rapid loss of fibrinolytic activity and did not approximate the required in vitro clot dissolution time.

Prolongation of the activity within the anterior chamber was considered next. Aqueous humor outflow was inhibited by the injection of human whole blood and excess bovine thrombin into the anterior

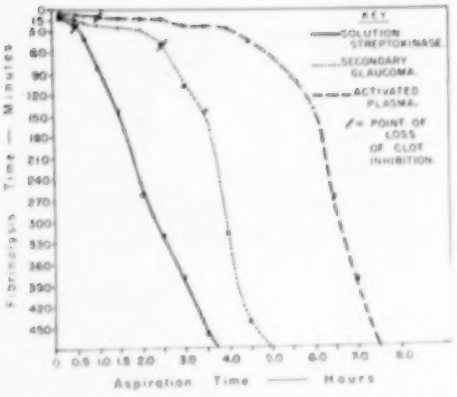

Chart 2 (O'Rourke). Comparative duration of anterior chamber fibrinolytic function.

chamber after paracentesis. A condition of acute secondary glaucoma developed in each eye within one hour.

Forty-eight eyes were so injected; 48 opposite eyes served as controls, and were subjected to simple paracenteses at this point. Two hours following these steps, tensions were recorded on all eyes using a Schiøtz tonometer. A second paracentesis was done and each eye injected with 0.2 cc. of streptokinase solution in concentration in 50,000 units per cc. The preliminary fibrinolysis time of this solution tested 10 separate times was an average of four minutes, two seconds.

At 30-minute intervals following these injections, aqueous humor was recovered from each of two glaucomatous eyes and two control eyes, and tested. The results of such are shown in Table 3.

Comparison showed a significant prolongation of fibrinolytic activity when aqueous humor outflow was impeded. It is interesting that in no case did streptokinase injection limit or retard the glaucomatous process instituted by the angle blocking with clotted human blood. Peripheral anterior synechias frequently seen in histologic sections may explain this.

It was evident that the streptokinase solution injected was rapidly dissipated by aqueous humor dilution and outflow. The effect of injecting a more viscous medium was studied. The possibility that plasma or serum proteins of large molecular size would not be removed so readily by aqueous humor outflow was considered. If such a plasma solution were activated by preliminary incubation with an excess of streptokinase prior to injection, prolonged activity could result.

As previously mentioned, plasma so treated will not clot. In vivo studies of such

TABLE 3

EFFECT OF SECONDARY GLAUCOMA ON AQUEOUS FIBRINOLYSIS TIME

Interval of Aqueous Humor Withdrawal *Time—Minutes	Tension (Schiøtz)		Fibrinolysis Time (minutes)		Clot Inhibition	
	Glaucoma	Control	Glaucoma	Control	Glaucoma	Control
15–	56	16	7.5	6.3	+	+
30–	44	14	8.6	18.8	+	+
45–	48	20	17.0	32.0	+	–
60–	32	18	19.0	84.0	+	–
90–	40	15	26.0	136	+	–
120–	60	19	30.0	260	+	–
150–	64	15	48.0	320	–	–
180–	32	20	110	380	–	–
210–	36	23	146	420	–	–
240–	44	18	318	Neg. 12 hr.	–	–
270–	64	15	440	Neg. 12 hr.	–	–

* Each time interval represents average values of four glaucomatous eyes (OS) and four control eyes (OD).

activity were done following injections of 0.2 cc. of activated plasma. Each of 70 rabbit eyes were so injected after paracentesis. At 30-minute intervals, specimens of aqueous humor were recovered from each of four eyes. Fibrinolysis time studies were done on 0.1 cc. portions of each specimen as described in the previous section.

As shown in Chart 2, a considerable prolongation of anterior chamber fibrinolytic activity resulted. Clearing of anterior chamber opacity occurred in about one hour, but significant fibrinolytic action remained between four and six hours. No tension increase was noted with this method. Clot inhibition ability was retained for two to five hours.

Section IV. Antistreptokinase activity

Early in his investigations, Tillet[29] was able to show an increase of serum antifibrinolytic activity in patients afflicted with hemolytic streptoccal infections. He speculated that the severity of the infection was related to the balance of antifibrinolytic versus fibrinolytic factors.[35]

Streptokinase is known to be antigenic: increase in specific anti-enzyme titer has followed its administration, clinically,[31, 36, 38] Guest[14] has shown such response to be present in animal sera in significant amounts.

Because such factors are of protein nature and are found in the blood the possibility of their presence in rabbit aqueous humor under conditions of the preceding studies was investigated. An effort was made to determine the influence such factors would exert on the concentrations of streptokinase employed. The following methods were used.

Twenty adult white rabbits were given bilateral anterior-chamber injections of streptokinase solution of 200,000 units per cc. concentration. The injections were repeated in seven days. At five-day intervals thereafter, aspirations of primary aqueous humor by paracentesis were done. Groups of eight eyes were aspirated on the 5th, 10th, 15th, 20th, and 25th day. Two hours after this, a second paracentesis was done on the same eyes to obtain the more viscid and often clotted plasmoid aqueous. Each aqueous sample was promptly tested for antistreptokinase activity according to steps described in Section II, E as antistreptokinase testing method.

This was essentially a test of the plasminogen activating ability of a standard concentration of streptokinase after its incubation with aqueous humor suspected to contain antistreptokinase activity. After the interaction of aqueous humor and streptokinase had been permitted, the mixture was added to an equal amount of plasma to be activated. The degree of activation of the plasma sample, as evidenced by its fibrinolysis time, was taken as a measure of antistreptokinase activity of the aqueous humor. Eight controls in which saline replaced the aqueous humor were simultaneously incubated for each time interval.

The results plotted in Chart 3 indicate a significant increase of antistreptokinase activity present in the plasmoid aqueous humor beyond the five-day interval. Primary aqueous humor showed little such activity. It appeared that high concentrations

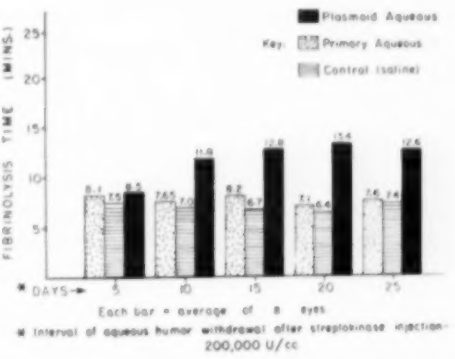

Chart 3 (O'Rourke). Prolongation of fibrinolysis time by antibody factor.

of streptokinase such as comprised the intraocular dose used here were capable of eliciting an antigenic serum response. This response was demonstrable in the plasmoid aqueous. Such activity was not present before the five-day period.

The antigenic dose injected here was five times greater than the contemplated therapeutic dose of streptokinase. The development of antibodies required about five days to become manifest. Such results might indicate an objection to repeated injections. Similar studies done with concentration of 50,000 units showed no antistreptokinase activity demonstrable by these methods.

Section V. Treatment of experimental lesions

The final segment of this study dealt with treatment of experimental lesions of the anterior chamber. With the foregoing limitations in mind, lesions were created within the rabbit eye to produce a suitable substrate for streptokinase fibrinolysis.

Simple hyphemas were induced by the injection of whole human blood, as described in the section on methods. Eight series of 10 rabbits each, were so injected giving a total of 160 eyes of which 80 served as untreated controls. Series bearing odd numbers were subjected to aspiration of anterior-chamber contents, four hours after injection of the streptokinase. Those of even numbers were not so treated.

In all treated eyes the first step consisted in injection of 0.2 cc. of streptokinase solution of concentration 50,000 units per cc. An equal amount of sterile saline was injected into the oposite eye of each animal. These served as controls. The periodicity of treatment by injection or aspiration varied with each series according to the following scheme:

Series I. Simultaneous administration of streptokinase and blood with aspiration at four hours.

Series II. Simultaneous administration of streptokinase and blood without aspiration.

Series III. Administration of streptokinase at four with aspiration at eight hours.

Series IV. Administration of streptokinase at four hours without subsequent aspiration.

Series V. Administration of streptokinase at eight hours with aspiration at twelve hours.

Series VI. Administration of streptokinase at eight hours without subsequent aspiration.

Series VII. Administration of streptokinase at twenty four hours with aspiration at 28 hours.

Series VIII. Administration of streptokinase at twenty four hours without subsequent aspiration.

The four-hour interval between injection in Chart 4 showed definite shortening of clearing time in all series in which aspiration followed streptokinase injection. The saline-injected control eyes did not show a similar reduction of clearing times. Their organized anterior-chamber contents made aspiration difficult, unsatisfactory, and incomplete.

All eyes treated with streptokinase showed a reduced clearing time when compared to saline treated controls. The rapid clearing time of hyphemas induced by si-

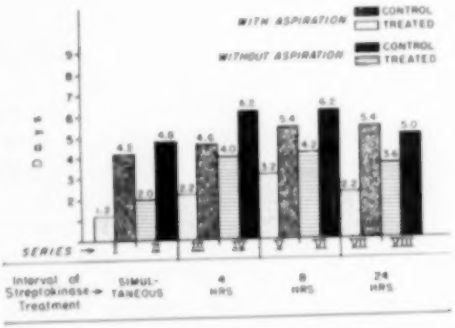

Chart 4 (O'Rourke). Treatment of hyphema with and without aspiration.

multaneous administration of streptokinase and blood may be due to the fact that the mixture failed to clot. This failure of the mixture to clot is due to the anticoagulant action of streptokinase in concentration exceeding 3,500 units per cc. when incubated with human plasma at body temperature for 10 minutes.

Section VI. Discussion and Summary

An attempt has been made to evaluate the enzyme activating agent, streptokinase, with regard to a possible therapeutic role for intracameral hemorrhage. Among the aspects studied were the development of analytic methods of the fibrinolytic function of aqueous humor. There followed in order consideration of toxicity, duration of action, prolongation of action, antigenic response, and finally the treatment of experimentally induced hyphemas.

Analytic methods carried out on primary and secondary aqueous were a necessary prelude to the latter group of studies. Techniques for the detection of free streptokinase and for active plasmin were described. Further preliminary studies served to confirm the necessity of employing human blood as a testing substrate.

In vitro studies of clot dissolution time demonstrated the increased rate of fibrinolysis associated with an increase of incubation temperature. This increase was greater than that obtained by raising the streptokinase concentration. The concentration of streptokinase appeared to play little part in changes of fibrinolysis time. It was not possible to corroborate the effects of increased temperature in experimental animals so the observation remains an in vitro one.

Toxic reactions noted appeared confined to the eye and were not observed below the concentration level of 50,000 units per cc. The changes observed were corneal edema with transient anterior uveitis. No permanent changes of any form were noted within the anterior segment and the duration of changes was in approximate proportion to the concentration injected. A similar though more severe response observed following injection of hyaluronidase suggested that this contaminent of the streptokinase preparation may be responsible for the toxic effects.

Preliminary studies have shown that a concentration of 50,000 units per cc. is sufficient for dissolution of an anterior chamber-sized clots in vitro, at body temperature.

Perfusion of the anterior chamber by aqueous flow appeared to remove injected streptokinase solution too rapidly for the desired effect. The prolongation of fibrinolytic activity that followed induced secondary glaucoma seemed to confirm the role of aqueous humor flow in this regard. Although the secondary glaucoma was induced by a thrombin and blood injection, no alleviation of the glaucomatous process followed streptokinase injection.

A significant prolongation of anterior chamber fibrinolytic function was effected by the injection of a viscid medium. Double-strength human plasma solution activated by incubation with streptokinase remains present within the chamber for over four hours. The amount of streptokinase in this mixture was innocuous. This mixture was not coagulable and therefore caused no obstruction to aqueous flow. No rise in intraocular pressure occurred with these intracameral injections in spite of the high protein contents. The presence of high protein level within the chamber may dispose to a reduction of intraocular pressure in a way analogous to eyes with anterior uveitis, according to Adler.[1]

Antistreptokinase factors could be demonstrated in the rabbit aqueous humor after the 12th day following initial streptokinase injection. They were only significant in plasmoid aqueous and did not appear likely to offset effectiveness of recommended therapeutic concentrations of streptokinase. No such antistreptokinase factors were demon-

strated in aqueous humor of animals previously unexposed to streptokinase.

A treatment series of experimental hyphemas with streptokinase with and without aspiration was evaluated. A slight reduction of clearing time was noted in eyes treated without subsequent aspiration. A more apparent reduction was evident in eyes that were aspirated because the partial dissolution of the clot rendered it more susceptible to aspiration. In general, the performance of streptokinase solution was not dramatic for the clearing of experimental hyphemata. A longer lasting agent is to be sought.

In spite of these observations, the possibility of anterior-chamber debridement by these enzymes is still theoretically plausible. This is based on the prolongation effect of the viscid protein medium. There is also the possibility that pure plasminogen may some day be available for activation by a minimum of free streptokinase. This would represent the maximum of fibrinolytic activity possible per unit weight. Extraction procedures for plasminogen isolation has been described[9, 10, 41] but were not successfully applied in this study.

Section VII. Conclusions

1. Clots of rabbit blood were not susceptible to fibrinolysis by streptokinase.
2. Human blood was the most suitable substrate for in vitro and in vivo testing.
3. Significant reduction of fibrinolysis time with increasing temperature was demonstrated in vitro.
4. Above a threshold level, increases of streptokinase concentration appeared to have little effect upon the fibrinolysis time of standard clots.
5. The anterior chamber of the rabbit eye tolerated concentrations under 50,000 units of streptokinase per cc.
6. The toxic effects which followed intracameral streptokinase injection, in concentration exceeding 50,000 units per cc., may be due to its hyaluronidase content.
7. Streptokinase solution failed to remain in significant concentration within the anterior chamber for longer than one hour after injection.
8. Streptokinase was retained in the anterior chamber up to tree hours by obstructing the outflow of aqueous humor.
9. Fibrinolytic activity was prolonged four to six hours by the intracameral injection of activated plasma.
10. The antistreptokinase factors could be demonstrated in plasmoid, but not in primary, aqueous humor 12 days after initial intracameral injection of streptokinase.
11. A slight reduction in clearing time of hyphemata was induced by anterior chamber streptokinase injection without associated aspiration.
12. The clearing time of hyphemas was further reduced by aspiration following streptokinase injection.

National Institute of Neurological Diseases and Blindness (14).

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Discussion

Dr. Alson E. Braley: About five years ago, when I was at Bellevue, I used streptokinase at the suggestion of Dr. Tillet and his group. We tried streptokinase in rabbits, and it produced very little inflammation in the anterior chamber of the rabbit eye in spite of the addition of the plasma factor from the human.

I put human blood into the anterior chamber of rabbits' eyes; and, although human blood does not remain too long in the anterior chamber of rabbits' eyes, it will remain for a time. Then, injecting streptokinase and streptodornase, and of course both go together along with a number of other impurities that Dr. O'Rourke has mentioned, we produced a very mild inflammation in the rabbit's eye, and were able to dissolve the clot that formed. However, the difference in the time of absorption of human blood from the anterior chamber of rabbit eyes was, to my mind, the same as the rate of absorption without the addition of streptokinase and streptodornase. At the insistence of Dr. Tillet we had five human eyes with various amounts of blood in the anterior chamber, one containing a good deal of pus, which was awaiting enucleation. I thought it worthwhile to inject streptokinase and streptodornase solution in concentrations never above 5,000 units of streptokinase and the equivalent of only about 200 units of streptodornase in the preparation that we had at that time.

All of these eyes resulted in a very severe secondary glaucoma, and we tried to relieve this secondary glaucoma by repeated paracenteses. Some of these paracenteses were done as many times as every hour in order to remove fluid from the eye. The reaction, I must say, was extremely severe, to the point that I would certainly hesitate ever to use streptokinase and streptodornase in the form that it is in at the present time in the anterior chamber of human eyes.

In spite of the very low concentration, the cornea became opaque and secondary glaucoma was very severe.

The next thing I tried streptokinase and streptodornase for was in lacrimal sacs, in which there was a good deal of fibrin-like material in the sac. Streptokinase was injected again in low concentration in an attempt to irrigate it out. This also resulted in a very severe reaction, and in one case the sac pointed and had to be opened externally.

Where have I had the best results with streptokinase and streptodornase? I have used it in a number of cases of conjunctivitis—the most successful are membraneous conjunctivitis cases, in which there is a membrane on the surface. The streptokinase will dissolve the membrane and many times will maintain it at a fluid level for quite a while. Some of you may remember some of the cases of conjunctivitis in which streptokinase and streptodornase were used.

It is a susbtance derived from the streptococcal organism. It produces a very severe reaction when injected into any cavity. It produces some reaction when applied to the surface, but it may have some antigenic properties. I felt that its reaction was so severe in the human that probably it should not be used in the eyes of human beings in its present form.

I have enjoyed hearing this paper. I was not sure from the paper whether Dr. O'Rourke used rabbit eyes or human eyes to do his experimental work. I presume it was rabbit eyes, from the amount of reaction he got.

Dr. O'Rourke (closing): I want to thank Dr. Braley for his helpful criticism and remarks. I confess to being unaware of his prior experience with intraocular streptokinase.

Our studies as completed within the rabbit eye led us to believe the agent too irritating for use within the human eye. Anticipated future work will include an attempt to activate the plasmin-containing euglobulin fraction of plasma. Such an activated isolated fraction may represent maximal plasmin fibrinolytic activity as initated by a minimal, nonirritating amount of streptokinase. Beyond a low critical level, increase of streptokinase concentration affects only the rate but not the degree of plasminogen activation. Much of this is speculative, however, and we have no further thoughts on the subject at the present time.

As to the nature of our plasmin preparation. We had no plasmin preparation, as our attempts at fractionation of plasminogen from whole plasma were not successful. Our activiated plasma solution employed double strength powdered, human plasma (Sharpe and Dohme, Lyovac) which is supplied for use with thrombin in the preparation of physiologic glue. Preliminary incubation of this with streptokinase gave us a viscous plasma solution whose plasminogen was activited and well retained within the eye.

THE CYTOLOGY OF EXTERNAL OCULAR DISEASE*

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In recent years the study of isolated cells removed by the scraping of lesions in situ has received considerable attention. Papanicolaou[1] and his associates have made extensive studies of this exfoliative cytology, and Blank et al.[2] and Baldridge and Leeb[3] have described diagnostically reliable cytologic findings in smears taken directly from vesicular lesions. Cytologic studies on the eye date from the observation of Herbert,[4] in 1903 and 1907, that a conjunctival eosinophilia was constantly associated with vernal catarrh.

In 1946 one of us (P. T.)[5] presented before this society a preliminary report in which the literature of this subject was reviewed and evidence submitted of the value of cytologic analysis of conjunctival exudates in the differential diagnosis of ocular infections of both known and unknown etiology. In the present report the material offered in this earlier paper is analyzed and revised in the light of further experience. Also presented are a number of new observations on the cytology of ocular tumors and of certain recently recognized ocular infections such as the Greeley type of keratoconjunctivitis,[6] Newcastle disease conjunctivitis,[7] and several inflammations due to fungi.

MATERIALS AND METHODS

In the study reported herewith, ocular scrapings from cases observed in the external disease clinic of the University of California Hospital and in the course of private practice were subjected to examination. Each case was studied completely in accordance with our routine external disease workup which includes the analysis of blood-agar and mannitol-agar plate cultures, the microscopic examination of scrapings from the lid margins and conjunctiva, and the clinical examination of the lesions with the corneal microscope and slitlamp.

In our experience the Giemsa stain has proven to be the best stain for the preparation of scrapings for cytologic study. It was used routinely and was prepared and applied according to the technique described in the earlier paper.

OBSERVATIONS OF CELLULAR REACTIONS

In addition to all the known types of leukocytes, there were special cells (for example, goblet cells) (fig. 1), altered normal cells (for example, large, multinucleated epithelial cells, and keratinized epithelial cells) (fig. 2), and abnormal numbers of macrophages (fig. 3) that were found to have definite diagnostic significance.

SIGNIFICANCE OF A NEUTROPHIL REACTION

A neutrophil reaction is the most frequent conjunctival reaction to all but two bacterial infections, to all the infections due to viruses of the Chlamydozoaceae family (trachoma, inclusion conjunctivitis, lymphogranuloma venereum), and to such fungus diseases as streptothrix conjunctivitis secondary to canaliculitis, and nocardial and monilial corneal ulcers. Several diseases of unknown etiology (for example, erythema multiforme and Reiter's disease conjunctivitis) are also characterized by a polymorphonuclear cell

* From the Department of Ophthalmology and the Francis I. Proctor Foundation for Research in Ophthalmology, University of California, School of Medicine, San Francisco. This work was supported in part by funds from Mrs. E. S. Heller.

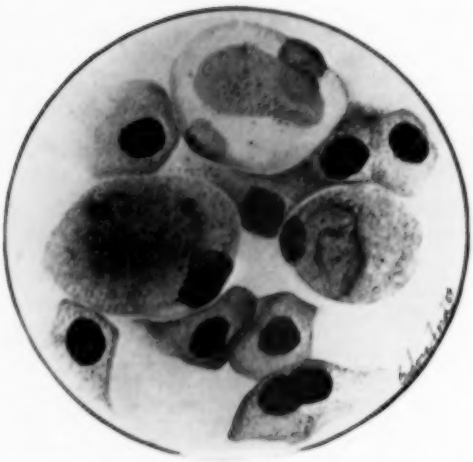

Fig. 1 (Kimura and Thygeson). Goblet cells from conjunctival scraping. Drawing from Giemsa-stained epithelial scraping.

exudate. In vernal conjunctivitis, a bacteria-free disease that is most certainly allergic in origin, there is a neutrophil reaction in addition to the characteristic eosinophilia.

It was noted in epidemic keratoconjunctivitis and herpetic keratoconjunctivitis that a mononuclear cell reaction would change to a predominantly polymorphonuclear cell reaction when a membrane formed.

Significance of an Eosinophil Reaction

The eosinophil is the characteristic cell of allergic inflammation. In the course of this study it was demonstrated in cases of vernal conjunctivitis, hay fever conjunctivitis, and allergic conjunctivitis due to various drugs, cosmetics, and other sensitizing antigens. A characteristic fragmentation of the eosinophil was observed in conjunctival scrapings from vernal conjunctivitis. Although not a pathognomonic sign, this fragmentation is valuable in differentiating vernal conjunctivitis from hay fever conjunctivitis in which it rarely occurs; when coupled with the clinical signs of vernal conjunctivitis, that is, the presence of giant papillae and the absence of hay fever, it may in fact be diagnostic. The free eosinophilic granules are more abundant in severe cases of vernal conjunctivitis than in mild cases.

Eosinophils were demonstrated in atropine-sensitivity conjunctivitis but not in eserine or pilocarpine conjunctivitis. This would seem to suggest strongly that the latter two types are toxic rather than allergic.

Significance of a Basophil Reaction

Basophils (fig. 4) in conjunctival scrapings and smears are significant only when seen in large numbers. Like eosinophils they

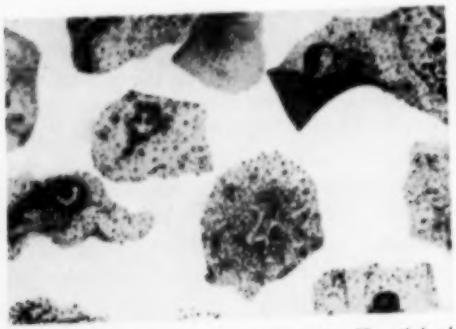

Fig. 2 (Kimura and Thygeson). Keratinized epithelial cells from conjunctival scraping. Drawing from Giemsa-stained epithelial scraping.

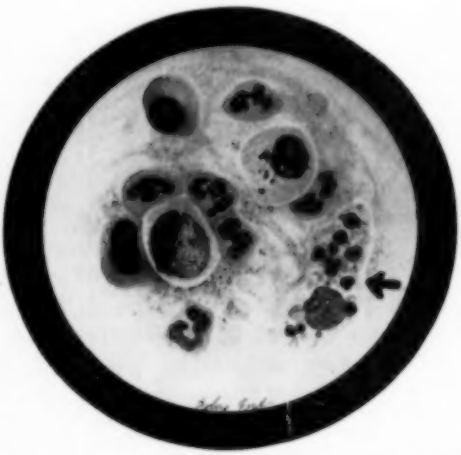

Fig. 3 (Kimura and Thygeson). Macrophage (Leber cell) from trachoma scraping (arrow). Drawing from Giemsa-stained scraping.

are characteristic of allergic inflammation, and particularly of vernal conjunctivitis; in scrapings from this disease they are occasionally more abundant than the eosinophils.

Significance of a mononuclear-cell reaction

Mononuclear cells are the predominant cells in the conjunctival scrapings from conjunctivitis due to, or suspected of being due to, a typical virus. In the absence of secondary infection, an almost 100-percent mononuclear cell response, predominantly lymphocytic, is found in epidemic keratoconjunctivitis, herpetic keratoconjunctivitis, and the acute follicular conjunctivitis of Béal.

Two recently described virus diseases of the eye, Newcastle disease conjunctivitis and the "Greeley disease," both produce a mononuclear cell exudate.

The cells comprising the mononuclear cell exudate were found to differ from disease to disease. The predominant cells were small lymphocytes (fig. 5) in some diseases, monocytes and large lymphocytes in others. The diagnostic significance of these differences, if any, has yet to be determined. We are investigating this point because of the presence of abnormally large lymphocytes ("virocytes") in the blood of patients with various systemic virus diseases.[8]

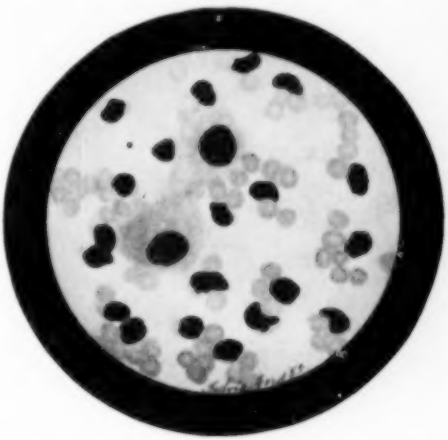

Fig. 5 (Kimura and Thygeson). Small lymphocytes. Drawn from conjunctival scrapings from acute follicular conjunctivitis of Béal.

Significance of plasma-cell reaction

Plasma cells are present in conjunctival scrapings from trachoma (fig. 6), and with an occasional exception from trachoma only. Why they are not seen more frequently in

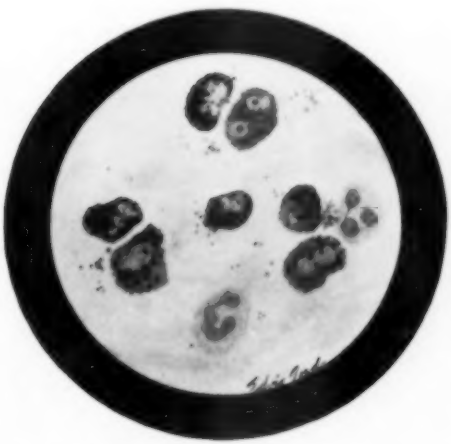

Fig. 4 (Kimura and Thygeson). Basophils. Drawn from Giemsa-stained conjunctival scrapings from vernal conjunctivitis.

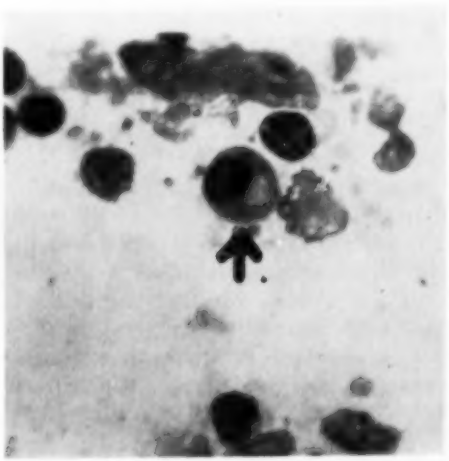

Fig. 6 (Kimura and Thygeson). Plasma cells (arrow). Photograph from conjunctival scraping.

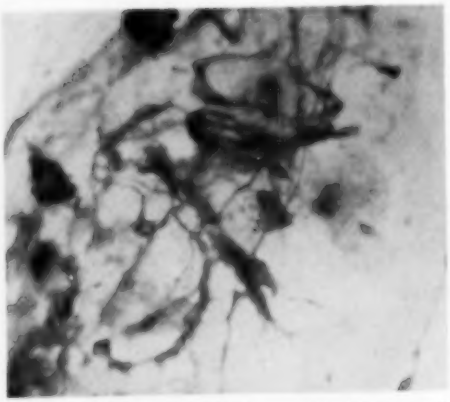

Fig. 7 (Kimura and Thygeson). Photograph of conjunctival scraping from keratoconjunctivitis sicca, showing keratinizing epithelial cells and mucous strands.

other conjunctival scrapings is indeed a mystery. For some unexplained reason they do not cross the epithelial barrier except in connection with the spontaneous rupturing of the follicles that occurs in trachomatous necrosis. As noted by Papanicolaou,[1] this is in sharp contrast to the almost constant appearance of plasma cells subepithelially in chronic infections.

Significance of Epithelial Changes

Keratinization of conjunctival epithelial cells (fig. 2) is always pathologic. When keratinized cells are seen in conjunctival scrapings they furnish diagnostic evidence of vitamin-A deficiency, exposure, cicatrization, keratoconjunctivitis sicca, or epithelial plaque.

Keratoconjunctivitis sicca is part of a generalized dryness of the mucous membranes of the body. The epithelium is never fully keratinized as it is in vitamin-A deficiency, nor is there an overgrowth of Corynbacterium xerosis on the cells. The epithelial cells from an advanced case contain keratin granules in the cytoplasm and there are degenerative changes in the nuclei (fig. 7). Scrapings contain an abnormally large number of goblet cells and usually a large amount of mucus. If there is secondary infection, polymorphonuclear leukocytes are also present.

Epithelial plaques are localized conjunctival lesions in which an excess of cells has undergone surface keratinization to form a thick horny layer of flattened cells. Keratinized epithelial cells are therefore also characteristic of scrapings from conjunctival epithelial plaques. Three cases of epithelial plaque of the bulbar conjunctiva, simulating in each case a Bitot's spot, were observed in the course of this study.

Differentiation from the Bitot's spot of vitamin-A deficiency was made on the basis of (1) absence of other signs of vitamin-A deficiency and (2) absence of therapeutic response to vitamin-A administration. Epithelial scrapings from the lesions contained keratinized epithelium and numerous diphtheroid bacilli. The cytologic picture was identical with that of the hyperkeratotic lesions of vitamin-A deficiency. In the two cases in which the plaques were removed surgically, microscopic examination confirmed the diagnosis.

Large, multinucleated epithelial cells (fig. 8) in conjunctival scrapings are characteristic of viral infection as opposed to bacterial infection. The same large cells have also been noted in scrapings from viral keratitis, for example, dendritic keratitis, but not

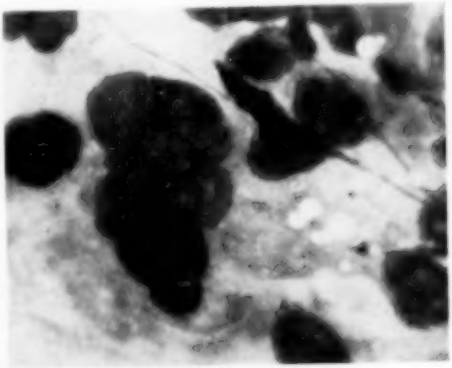

Fig. 8 (Kimura and Thygeson). Large multinucleated epithelial cells. Photograph of scrapings of herpetic keratitis.

in scrapings from bacterial keratitis. Although found most often in herpetic keratoconjunctivitis, these cells are not signficantly characteristic to indicate anything more specific than a viral etiology.

Pigment granules of two types (fig. 9), large and small, were noted in the epithelial cells of the conjunctiva. These are normal components and are seen more frequently in the pigmented races. To the uninitiated, however, they can easily be mistaken for inclusion bodies, engulfed bacteria, or rickettsial bodies. Unstained they are brown in color, but stained with Giemsa they assume a definite blue-green tinge and can be recognized easily and differentiated from other cell inclusions.

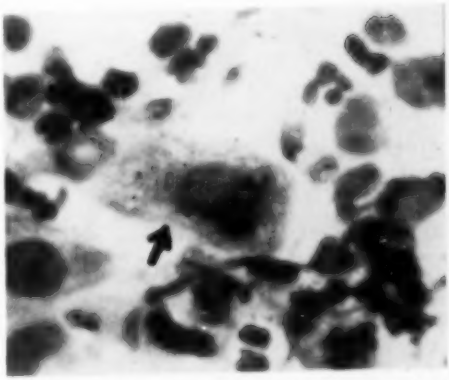

Fig. 9 (Kimura and Thygeson). Photograph of pigment granules in a conjunctival epithelial cell.

Cytology of corneal ulcers

In an earlier paper,[9] one of us (P. T.) reported on the etiology of corneal ulcers. It was shown that in almost every case an etiologic diagnosis could be based on the examination of scrapings from the advancing edge of the ulcer. The pneumococcus was in the past the most frequent cause of central corneal ulcer.

Since the advent of the sulfa drugs and the antibiotics, however, only a very few pneumococcic cases are seen and corneal ulcers are on the whole uncommon today. Probably as a result of the use of antibiotics, organisms of low virulence and pathogenicity, such as fungi, are now more frequently found to be causal.

Two cases of nocardial corneal ulcer following a corneal foreign body were diagnosed from an examination of scrapings taken directly from the advancing edge of the ulcer, and one case of monilial corneal ulcer was diagnosed in the same way. Both Nocardia and Monilia are low in virulence and have been almost unknown in the past as causes of corneal ulcer. Scrapings from all three cases showed an abundance of polymorphonuclear leukocytes in addition to the micro-organisms.

It is of interest that a neutrophil reaction was observed in scrapings from three cases of ulcer due to the Diplobacillus of Petit but not in scrapings from the definitely nonpyogenic conjunctivitis due to the Diplobacillus of Morax-Axenfeld. Scrapings from trachomatous corneal ulcers and catarrhal ulcers both produced polymorphonuclear cell reactions and scrapings from phlyctenular corneal ulcers a mononuclear cell reaction. As noted above, direct scrapings from herpes simplex corneal ulcers contained a significant number of multinucleated epithelial cells. Scrapings from a few cases of Mooren's ulcer were examined but the cytologic picture was in no way characteristic.

Cytology of external ocular tumors

Bowen's disease (intraepithelial epithelioma, carcinoma in situ) of the cornea and conjunctiva was subjected to cytologic examination. Abnormally large epithelial cells (fig. 10) were seen, many of them with signs of partial keratinization. One case had been treated as a keratitis of unknown etiology for five years. Biopsy confirmed the diagnosis of Bowen's disease.

Molluscum contagiosum. Curettings from a suspected molluscum contagiosum nodule were smeared on a slide, stained with Giemsa, and examined. Typical molluscum

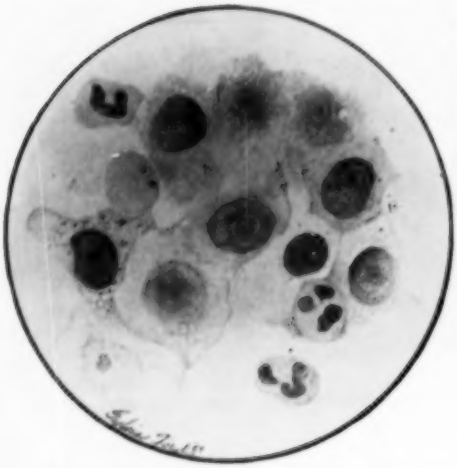

Fig. 10 (Kimura and Thygeson). Large epithelial cells. Drawn from direct scrapings from conjunctival Bowen's disease.

cells (fig. 11) filled with virus particles (fig. 12) were readily identifiable.

Infectious warts of the lid margin were scraped and the scrapings examined. The cytologic picture had no diagnostic value.

Squamous cell carcinoma scrapings contained only keratinized epithelial cells and had no diagnostic value.

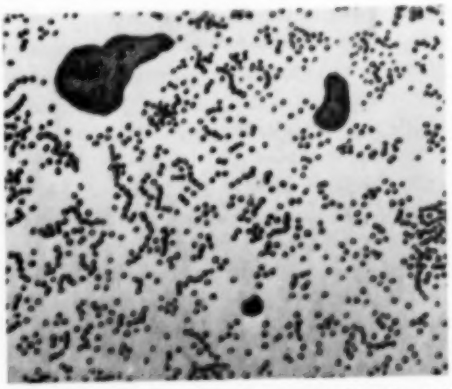

Fig. 12 (Kimura and Thygeson). Drawing of virus particles expressed from a molluscum lesion. Loeffler's flagella stain.

Pyogenic granulomas of the lid margins were scraped and the scrapings studied cytologically; they were loaded with polymorphonuclear leukocytes.

Conjunctival melanoma. Examination of direct scrapings from two cases of conjunctival melanoma revealed an abundance of large, melanin-containing cells (fig. 13). The nuclei of these cells were large and had very prominent nucleoli. The amount of melanin varied but the cytoplasm of most of the cells was loaded with large and small pigment

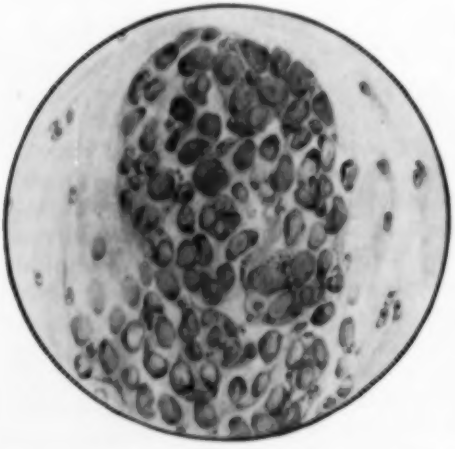

Fig. 11 (Kimura and Thygeson). Molluscum cells. Drawing of a histopathologic section.

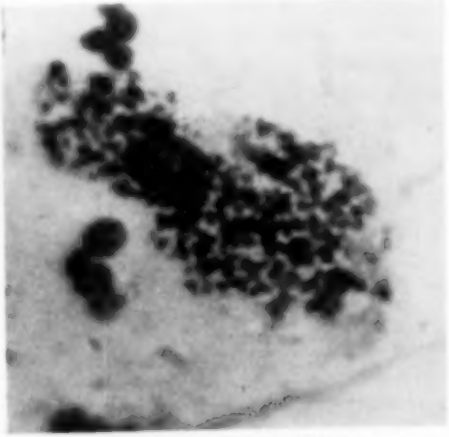

Fig. 13 (Kimura and Thygeson). Large melanin-containing cell. Photograph of scrapings from conjunctival melanoma.

particles. The diagnosis in both cases was evident clinically and confirmed histopathologically after surgical removal of the tumors.

Discussion

The results of this study substantiate the previous conclusion that except for Neisseria catarrhalis and diplobacillary (Morax-Axenfeld) infections of the conjunctiva, all bacterial infections produce a polymorphonuclear cell reaction. The viruses of the Chlamydozoaceae family were also pyogenic. Like the streptothrix conjunctivitis secondary to a canaliculitis, both nocardial and monilial keratitis were characterized by a polymorphonuclear cell reaction.

The demonstration of a mononuclear cell reaction is considered significant in diagnosing a typical virus infection or a disease probably due to a virus such as acute follicular conjunctivitis, Béal. Herpes-simplex virus, the virus of epidemic keratoconjunctivitis, and the virus of molluscum contagiosum all induced a lymphocytic reaction which had considerable diagnostic value when correlated with clinical and bacteriologic findings.

Very little is known about the function of lymphocytes and the mononuclear cells in general. Lymphocytes have very little phagocytic power and yet a great migration of them occurs in chronic inflammations. Round cell infiltration and regional lymphadenopathy are apparently concerned with antibody formation, and it has been suggested that the lymphocytes function as trephocytes,[10] that is, as cells supplying nutrition.

In our preliminary report the cytologic findings in conjunctival epithelial scrapings from cases of vitamin-A deficiency were regarded as diagnostically reliable in all cases. Since that paper was written, however, we have seen three cases of epithelial plaque of the conjunctiva which were unaffected by vitamin-A therapy but in which the cytologic picture was identical with that of vitamin-A deficiency. In fact no differentiation between these two conditions seemed possible on a cytologic basis.

Partially keratinized epithelial cells of the conjunctiva are a characteristic of keratoconjunctivitis sicca and highly specific. Similar partial keratinization of epithelial cells was also found in scrapings from two cases of Bowen's disease of the conjunctiva and cornea. These two diseases can be readily differentiated on the basis of clinical differences, however, and on the fact that in Bowen's disease there are neither goblet cells nor excessive mucus.

Typical molluscum cells, which we believe to be pathognomonic of molluscum contagiosum, are to be found in curettings of molluscum nodules. The reliability of this cell as a basis for diagnosis appears to be equal to the reliability of the histopathologic findings. In none of our cases of molluscum conjunctivitis have we been able to find molluscum inclusions in conjunctival scrapings, and from this we deduce that the conjunctivitis is either toxic or allergic. Molluscum nodules of the conjunctiva, although reported in the literature, have not been seen by us.

Repeated attempts to identify specific cell changes in lid margin scrapings from cases of herpes zoster, the common wart, and infectious papillomatosis of the conjunctiva have been unsuccessful.

The diagnostic value of cell inclusions has long been established and many searches for inclusions in preparations of exfoliated material from ocular disease have been made since the findings of Halberstaedter and Prowazek in 1907. In our studies only five types of inclusions have been identified in scrapings from ocular tissue, that is, the inclusions of trachoma, inclusion blennorrhea, vaccinia, molluscum contagiosum, and lymphogranuloma venereum. In herpetic keratoconjunctivitis we have failed consistently to find the intranuclear inclusion bodies in scrapings from the conjunctiva, cornea, or lids, even though they are easy to see in histopathologic sections. The chromatin of

the nucleus probably covers the intranuclear inclusion in the intact cell. We had hoped they would be demonstrable by special stains or phase microscopy but so far this has not been the case.

The inclusions of trachoma and inclusion blennorrhea are morphologically identical. Braley[11] noted that more of the inclusions of inclusion blennorrhea are to be found in scrapings from the lower tarsal conjunctiva than from the upper, and that in trachoma more are to be found in scrapings from the upper tarsal conjunctiva than from the lower. We have confirmed this finding and regard it as a valuable laboratory aid in the differentiation of the two diseases.

In considering other indications of virus activity we have noted a characteristic lymphocytosis or monocytosis in conjunctival viral infection. There have also been more multinucleated epithelial cells in scrapings from viral diseases than in scrapings from bacterial infections. The significance of this is not as yet clear. In mucous membrane scrapings from herpetic keratoconjunctivitis we have searched unsuccessfully for the balloon cells which Blank[2] reported finding in herpetic vesicles.

Conclusions

1. The present study amends and extends our previous report of the cytology of ocular disease and indicates that cytologic examination of conjunctival scrapings and exudates should be part of the routine examination of every eye infection.

2. Cytologic examination of ocular tumors was rewarding and should be further investigated.

3. The recently described virus diseases, that is, "Greeley disease" and Newcastle-disease conjunctivitis, both produce a mononuclear cell reaction.

The Medical Center (22).
220 Meridian Road (26).

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Discussion

Dr. Alson E. Braley: This is an excellent piece of work, and should emphasize to all of you the importance of scrapings of the conjunctiva and the cornea in cases of conjunctivitis, keratitis, or any inflammation in that region.

I would like to put on record for your information some additional evidence of how important scrapings from a corneal ulcer may be.

A number of years ago I was referred a patient in New York who had a corneal ulcer that had been present for approximately four months. Scrapings from the ulcer showed a most peculiar histology. There were a few polys, not many, and a great many epithelial cells. However, there was the most peculiar-shaped organism present, one with which I was not familiar. It turned out to be

a specific type of diatome that occurs in only one area of the world, and that is on the White Cliffs of Dover.

This diatome is characteristic, and I had to look in a number of books in order to find it. The first thing I thought of was that it came from the water; but since it occurred only on the White Cliffs of Dover (it had a nucleus and sharp spines that were very characteristic), I asked the man if he had ever been to England. He said yes, and that he had been telling all the doctors ever since he got it, but he hadn't told me because they wouldn't believe him and he thought I wouldn't, either.

He said they landed at Southampton. He said, "I thought I got something in my eye while I was taking a walk along the beach. That was the beginning of my corneal ulcer." No one would pay any attention to that history because they couldn't find any evidence of a foreign body.

If a simple scraping had been taken, a diagnosis could have been made. The use of 1/5,000 copper sulfate as solution drops in the eye cured the ulcer that had been present for several months.

I believe scrapings probably are more important than cultures from the conjunctiva. They give the ophthalmologist a great deal more information about what is present in the eye than he gets many times from cultures that may be taken any place.

I would like to congratulate Dr. Kimura and Dr. Thygeson on these excellent additions to the importance of scrapings from the conjunctiva, and emphasize to you the necessity of continuing to take scrapings and to look at them for the presence of many organisms.

DR. JAMES H. ALLEN: I would like to emphasize Dr. Braley's compliments to the authors. I think they have done a beautiful piece of work.

I would like to add a few points that I think are of practical importance. First, I would like to emphasize the importance of making these examinations when the patient is first seen and not after a series of treatment failures. If the examinations are made before the patient has had any treatment, it makes the diagnosis and the differential diagnosis much simpler.

I also would like to emphasize a point which I believe the authors made, and that is the importance of the ophthalmologist making the examination of the secretion smears and scrapings. Most of the laboratories around the country are willing to do the work but on the average their interpretations of secretion smears and scrapings from the eye are rather poor. Until one can train a technician to recognize the relative importance of the various features emphasized by Dr. Kimura today, it is much better to examine the material oneself.

Finally, I would like to point out a couple of causes of confusion in the recognition of virus inclusion bodies. One of these is pigment granules in epithelial cells. We see a number of highly pigmented individuals, and very dark-complexioned persons, in my part of the country. Very frequently pigmented epithelial cells are found in scrapings from their eyes. The uninitiated have a tendency to call these inclusion bodies. They are inclusions, but not virus inclusions.

Another important thing which I believe causes confusion with virus inclusion bodies is phagocytic mononuclear cells. When found in cases of trachoma they are referred to as Leber cells. However, we have been finding these cells in small numbers in an increasing number of chronic inflammatory lesions of the conjunctiva. On several occasions I have been asked if cells of this type were not cells containing virus inclusion bodies.

While I do not believe these cells are pathognomonic of trachma, I would like to emphasize the point Dr. Thygeson has made on repeated occasions, that an abundance of phagocytic mononuclear cells is highly suggestive of trachoma.

FURTHER STUDIES ON THE NATURE OF THE EXOPHTHALMOS-PRODUCING PRINCIPLES IN PITUITARY EXTRACTS*

GEORGE K. SMELSER, PH.D., AND V. OZANICS, M.S.
New York

Although it has been assumed by many that both clinical and experimental exophthalmos are caused by the thyrotrophic hormone, no proof of this assumption has been forthcoming. Indeed, evidence requiring modification of this idea has been presented[1] showing that at least two anterior pituitary factors are involved in the production of experimental exophthalmos, one of these probably being adrenocorticotrophic hormone (ACTH), and the other possibly the thyroid-stimulating hormone (TSH). A recent report that thyroid-stimulating hormone is not involved in the production of exophthalmos in fish will be discussed later.

It has been shown[1] that relatively pure anterior pituitary preparations of growth hormone, prolactin (luteotrophin), ACTH, gonadotrophins (follicle-stimulating hormone—FSH, and luteinizing-hormone—LH or ICSH), do not, when injected alone, cause exophthalmos, edema, and enlargement of the orbital fat or hypertrophy of the Harderian gland. However, in view of experiments showing that exophthalmos results from the action of more than one pituitary factor, it is possible that, though not active alone, these hormones may be involved in exophthalmos when present in combination with others.

The evidence for this was drawn from experiments in which exophthalmos and orbital modification in guinea pigs resulted from the combined injection of ACTH and crude anterior pituitary preparations, whereas, no exophthalmos was produced when these materials were given alone.

It was concluded that some substance in the ACTH preparation (probably ACTH itself) potentiated the exophthalmogenic material in the crude pituitary extract. The identity of the potentiating agent and of the active substance in the crude extract is the subject of the present report.

METHODS

The experiments were conducted as described previously.[1] Young female albino guinea pigs (280 to 300 gm.) obtained from a single dealer were thyroidectomized 10 days prior to the first administration of the hormone preparations. The subcutaneous injections were given daily for 12 days in aqueous solution, with the exception of cortisone (Cortone acetate, Merck) and ACTH gel (Armour), which were given as supplied by the company. The animals were killed the day following the last injection and a complete autopsy was performed.

Vernier caliper measurements of the distance from the skull, at the supraorbital notch, to the limbus is the value given in the charts to indicate exophthalmos. In addition, the greatest distance between the apices of the corneas, together with the interorbital measurement of the skull, was also recorded. All measurements were made post mortem after the lids had been removed and the orbital vessels emptied.

The orbital contents were dissected, the fat, muscles, and glands weighed separately, fixed for histologic examination, and the degree of edematous infiltration of the fat, characteristic of exophthalmos, noted. The autopsy data are shown in the charts.

Except for ACTH, all of the hormone preparations† used were assayed as an inte-

* From the Departments of Ophthalmology and Anatomy, Columbia University College of Physicians and Surgeons.

† We wish to express our thanks for the cooperation of Dr. Sanford L. Steelman of the Biochemical Research Department of the Armour Laboratories, and for the generous supply of the

TABLE 1

ACTIVITY-EQUIVALENT STANDARDS

Preparations	Daily (mg.)	Dosage (TSH units*)		Standard Beef Pituitary Extract (mg.)
Growth Hormone[1]	5.0	1.5	Containing growth activity equivalent to	80.0
Growth Hormone[2]	2.0	1.0	Containing growth activity equivalent to	50.0
Thyrotrophic Hormone[3]	2.5	50	Containing thyrotrophic activity equivalent to	5.0
Horse anterior pituitary	8.0	1.0	Containing thyrotrophic activity equivalent to	0.1
Gonadotrophin[4]	6.0	1.0	Containing gonadotrophic activity equivalent to	10.0
Gonadotrophin[5]	4.0	1.0	Containing gonadotrophic activity equivalent to	40.0
Prolactin[6]	2.0	1.0	Containing prolactin activity equivalent to	40.0
ACTH Gel[7]	5 U	0.25	Containing ACTH activity equivalent to	40.0

* One TSH unit is the minimal substance causing hypertrophy of the chick thyroid and is equivalent to 1.0 mg. of U.S.P. thyrotrophin reference substance containing 0.05 mg. U.S.P. thyrotrophin unit which has been obtained through the courtesy of Nr. Nichols of the U.S.P. Reference Standards, U.S. Pharmacopoeial Convention, Inc.

[1] Armour growth hormone BGH 369-265 (beef origin).
[2] Organon growth hormone Res. P. 279.
[3] Organon thyrotrophic hormone No. 17510, equivalent in potency to the U. S. P. standard.
[4] Armour pituitary gonadotropin (F.S.H.) K 45308 R (pig origin).
[5] Armour pituitary gonadotropin #317-115 (horse origin).
[6] Squibb Prolactin 71713, having a prolactin potency of 20–25 I.U. per mg.
[7] Armour H.P. ACTHAR Gel #M21305, prolonged action, 40 Armour units per cc.

gral part of the experiment. These tests also were carried out as described earlier and the preparations were assayed against a standard beef pituitary extract; the various activity equivalents to it are stated in Table I. The exophthalmos-producing activity of this standard had been determined earlier and its potency in this respect is given in the charts. The standard beef and horse pituitary extracts were made in the laboratory as described previously, and are identical with those used in that publication.[1]

Several groups of experiments were devised and are described under the following headings:

I. *The nature of the potentiating agent in the ACTH preparation.*

II. *The nature of the material in pituitary extracts which, together with the potentiator, causes exophthalmos.*

1. The rôle of growth hormone, prolactin, gonadotrophins in the etiology of exophthalmos.
2. The effect of small quantities of thyroid-stimulating hormone on exophthalmos.
3. The effect of combinations of pituitary hormones in the production of exophthalmos.

EXPERIMENTAL

I. NATURE OF POTENTIATING AGENT

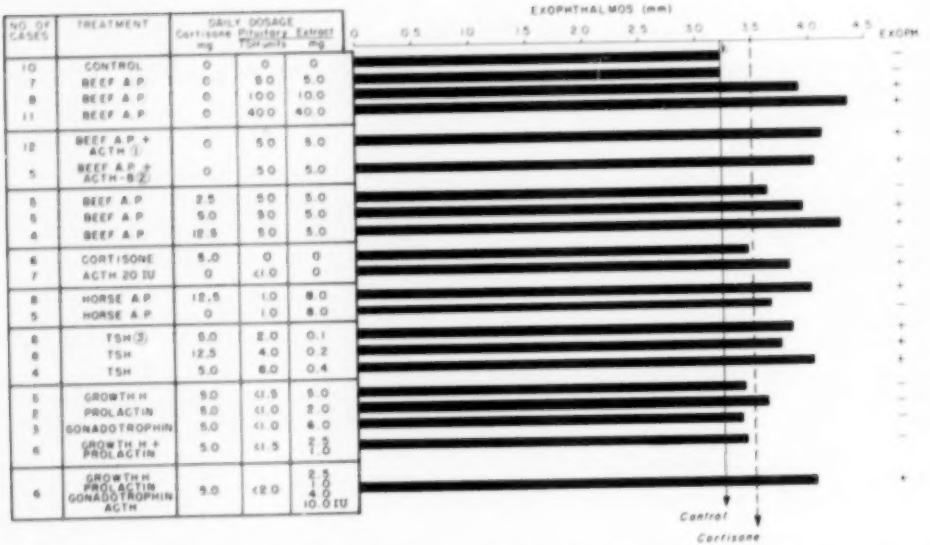
Twelve thyroidectomized guinea pigs were injected with a combination of five units of ACTH in gelatin (Armour) and five mg. of the standard beef pituitary extract daily, which resulted in a good exophthalmos and orbital changes, as have been described. Five mg. of this same standard extract did not cause exophthalmos, nor did this quantity of

Armour products which were used in these experiments.

We are indebted to Dr. K. W. Thompson of Organon, Inc., for his contribution of thyrotrophic and growth hormones.

The prolactin preparation used in this experiment was obtained through the courtesy of Dr. A. Borman of E. R. Squibb and Sons.

The ACTH-B sample 52R6239 was received through the kindness of Dr. Norman G. Brink of the Research Division of Merck and Company, Inc.



NO. OF CASES	TREATMENT	DAILY DOSAGE Cortisone mg	Pituitary Extract TSH units	Pituitary Extract mg
10	CONTROL	0	0	0
7	BEEF A.P.	0	5.0	5.0
8	BEEF A.P.	0	10.0	10.0
11	BEEF A.P.	0	40.0	40.0
12	BEEF A.P. + ACTH (1)	0	5.0	5.0
5	BEEF A.P. + ACTH-B (2)	0	5.0	5.0
5	BEEF A.P.	2.5	5.0	5.0
5	BEEF A.P.	5.0	5.0	5.0
4	BEEF A.P.	12.5	5.0	5.0
6	CORTISONE	5.0	0	0
7	ACTH 20 IU	0	<1.0	0
8	HORSE A.P.	12.5	1.0	8.0
5	HORSE A.P.	0	1.0	8.0
8	TSH (3)	8.0	2.0	0.1
8	TSH	12.5	4.0	0.2
4	TSH	5.0	8.0	0.4
5	GROWTH H	5.0	<1.5	5.0
2	PROLACTIN	5.0	<1.0	2.0
3	GONADOTROPHIN	5.0	<1.0	6.0
6	GROWTH H + PROLACTIN	5.0	<1.5	2.5 7.0
6	GROWTH H PROLACTIN GONADOTROPHIN ACTH	5.0	<2.0	2.5 1.0 4.0 10.0 IU

Chart 1 (Smelser and Ozanics). Effect of cortisone on the orbital weights and exophthalmos measurements. (Five units/day Armour high potency ACTHAR Gel M21305 prolonged action. All ACTH used in these experiments was of this type unless otherwise noted. Fifty to 100 gamma/day Merck ACTH-B Corticotropin No. 52R6239 in a vehicle of 16-percent gelatin. Thyrotrophic hormone, Organon No. 17510 equivalent in potency to the U.S.P. standard substance.)

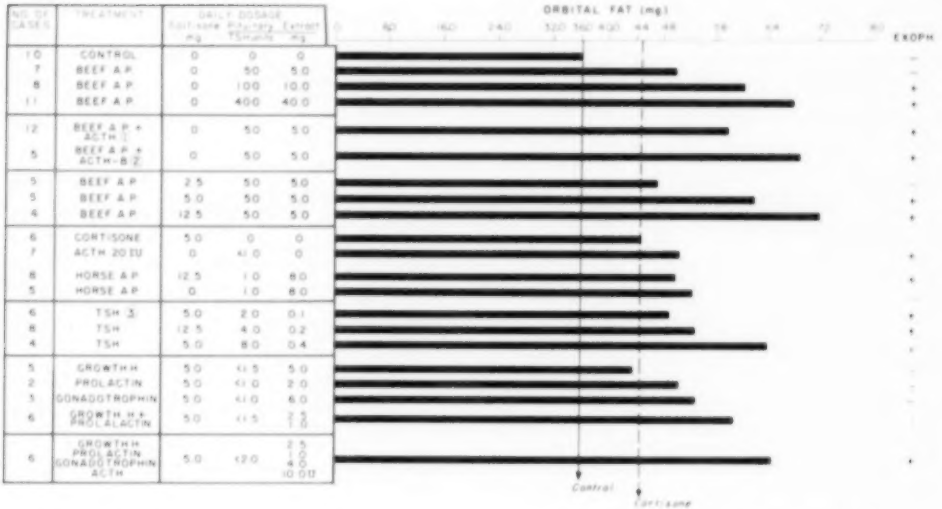
ACTH when given alone. This experiment was repeated with a more highly purified ACTH-B, which was found by assays, in the Merck Laboratories, to possess about 150 times the activity of the Armour standard material. The method of its preparation has been reported.[2,3]

Daily injection of 50 to 100 gamma of ACTH-B, together with five mg. of the standard extract, produced an excellent exophthalmos and even more marked hypertrophy of the orbital contents than did the original ACTH gel preparation, thus indicating that the potentiating agent is, in fact, ACTH. Presumably, the latter acts by stimulating the production of adrenal steroids, which would be the material directly acting synergistically with the pituitary extract to produce exophthalmos.

Cortisone was selected as an example of such a steroid and 2.5, 5.0, or 12.5 mg. were given daily together with five mg. of the standard beef extract into a group of 14 test guinea pigs. Exophthalmos resulted with all three doses of cortisone and was greater when the larger amounts were given. The modification of the orbital fat was striking, but that of the Harderian gland much less so.

It appears evident that the potentiating agent in the ACTH preparation is ACTH itself, and that it acts by increasing the production of some adrenal steroid such as cortisone. The possibility that either cortisone or ACTH might act directly on the orbit was tested by treating a series of guinea pigs with large doses of either cortisone or ACTH alone. Two groups of six guinea pigs each were injected with 5.0 or 12.5 mg. cortisone, and a third group of seven guinea pigs was treated with 20 units ACTH daily for 12 days. These amounts, 32.0 mg. cortisone/kg. body weight/day and 50 units ACTH gel/kg. body weight/day were considered heavy doses and are more than twice that given in most of the other experiments.

A slight increase in the protrusion in the



NO. OF CASES	TREATMENT	DAILY DOSAGE Cortisone mg	Pituitary Extract mg	ACTH mg
10	CONTROL	0	0	0
7	BEEF A.P.	0	50	5.0
8	BEEF A.P.	0	100	10.0
11	BEEF A.P.	0	400	40.0
12	BEEF A.P. + ACTH-C	0	50	5.0
5	BEEF A.P. + ACTH-B Z	0	50	5.0
5	BEEF A.P.	2.5	50	5.0
5	BEEF A.P.	5.0	50	5.0
4	BEEF A.P.	12.5	50	5.0
6	CORTISONE	5.0	0	0
7	ACTH 20 IU	0	40.0	0
8	HORSE A.P.	12.5	1.0	8.0
5	HORSE A.P.	0	1.0	8.0
6	TSH 3.	5.0	2.0	0.1
8	TSH	12.5	4.0	0.2
4	TSH	5.0	8.0	0.4
5	GROWTH H.	5.0	0.5	5.0
2	PROLACTIN	5.0	4.0	2.0
3	GONADOTROPHIN	5.0	4.0	6.0
6	GROWTH H. + PROLACTIN	5.0	0.5	2.5 1.0
6	GROWTH H. PROLACTIN GONADOTROPHIN ACTH	5.0	12.0	2.5 1.0 4.0 10.00

Chart 2 (Smelser and Ozanics). Effect of cortisone on the orbital fat and exophthalmos measurements. (Five units/day Armour high potency ACTHAR Gel M21305 prolonged action. All ACTH used in these experiments was of this type unless otherwise noted. Fifty to 100 gamma/day Merck ACTH-B Corticotropin No. 52R6239 in a vehicle of 16-percent gelatin. Thyrotrophic hormone, Organon No. 17510 equivalent in potency to the U.S.P. standard substance.)

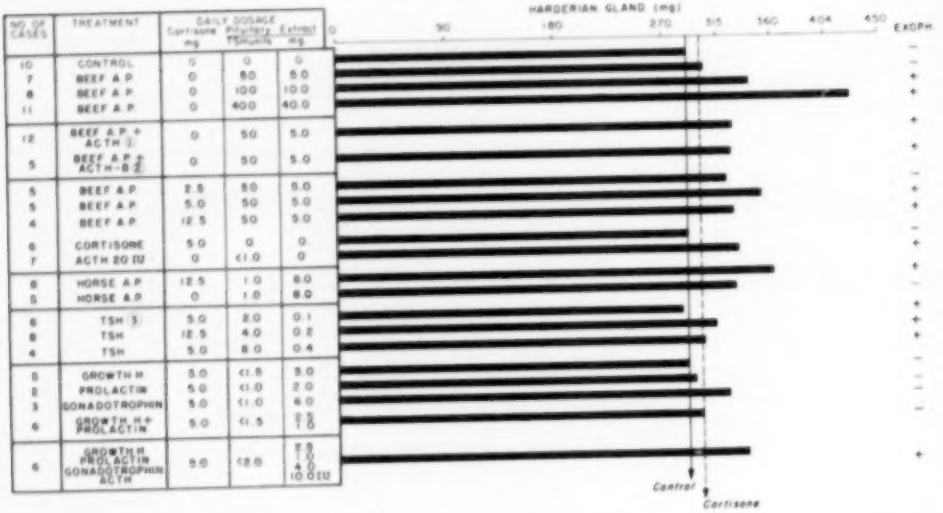
globe was obtained with both materials; the orbital fat was somewhat hypertrophied, and there was a minimal increase in the weight of the Harderian gland. ACTH was more effective than the larger dose of cortisone in all three respects; however, in no case was a clear-cut exophthalmos obtained, although a tendency in this direction was noted. The effect of cortisone on the orbital weights and exophthalmos measurement is indicated by a dotted line on Charts 1 to 3.

II. Nature of the potentiated substance

It is evident that small quantities of the standard beef pituitary extract will produce exophthalmos when administered in conjunction with ACTH or cortisone, but, since all known pituitary factors are present in this extract, whether one of these or some other factor is exophthalmogenic is unknown. In earlier studies separate pituitary hormones were tested for their ability to produce exophthalmos, but not in conjunction with ACTH or corticosteroids.

A series of experiments were therefore carried out in which relatively pure preparations of either growth hormone, prolactin, gonadotrophins, or thyrotrophin were injected together with five mg. cortisone. Cortisone was chosen as the potentiating agent, since the problem of contamination with pituitary factors does not arise as it would if ACTH were used.

Prolactin and two different preparations each of growth hormone and gonadotrophins were used—one of the latter was prepared from pig, and the other from horse pituitary glands and differed in the relative amount of follicle-stimulating hormone and luteinizing hormone they contained. The potency of each preparation in relation to the standard beef extract, and the degree of contamination with thyroid-stimulating hormone, is given in the table. The quantity of each hormone given per day contained two to 16 times that of the particular physiologic activity of five mg. of the standard beef preparation. Therefore, if exophthalmos was due to the interaction of cortisone with either the growth hormone, prolactin, or the



NO. OF CASES	TREATMENT	DAILY DOSAGE Cortisone mg	Pituitary TSH units	Extract mg
10	CONTROL	0	0	0
7	BEEF A.P.	0	80	5.0
8	BEEF A.P.	0	100	10.0
11	BEEF A.P.	0	400	40.0
12	BEEF A.P. + ACTH (1)	0	50	5.0
5	BEEF A.P. + ACTH-B (2)	0	50	5.0
5	BEEF A.P.	2.5	50	5.0
5	BEEF A.P.	5.0	50	5.0
4	BEEF A.P.	12.5	50	5.0
6	CORTISONE	5.0	0	0
7	ACTH 20 IU	0	<1.0	0
8	HORSE A.P.	12.5	1.0	8.0
5	HORSE A.P.	0	1.0	8.0
6	TSH (3)	5.0	2.0	0.1
8	TSH	12.5	4.0	0.2
4	TSH	5.0	8.0	0.4
5	GROWTH H	5.0	<1.5	5.0
5	PROLACTIN	5.0	<1.0	2.0
3	GONADOTROPHIN	5.0	<1.0	4.0
6	GROWTH H + PROLACTIN	5.0	<1.5	5.0 2.0
6	GROWTH H PROLACTIN GONADOTROPHIN ACTH	9.0	<2.0	5.0 2.0 4.0 10.0 IU

Chart 3 (Smelser and Ozanics). Effect of cortisone on the Harderian gland and exophthalmos measurements. (Five units/day Armour high potency ACTHAR Gel M21305 prolonged action. All ACTH used in these experiments was of this type unless otherwise noted. Fifty to 100 gamma/day Merck ACTH-B Corticotropin No. 52R6239 in a vehicle of 16-percent gelatin. Thyrotrophic hormone, Organon No. 17510 equivalent in potency to the U.S.P. standard substance.)

gonadotrophin contained in the standard extract, it would certainly also be produced when these hormones were given singly with cortisone.

The results of these experiments are summarized in the charts. In no case was exophthalmos nor unequivocal changes in the fat or Harderian gland evoked. Since similar results were obtained, data from the experiments with only one growth hormone and one gonadotrophin are shown in the charts.

Both prolactin and the gonadotrophin K-45308R produced some hypertrophy of the orbital fat over that caused by the cortisone injected controls. In these few cases the modification was in the direction of exophthalmos (that is, slight edema and hypertrophy), but the change was not great and certainly not in proportion to the prolactin or gonadotrophic activity contained in the extracts. These experiments very clearly show that the exophthalmogenic factor in the standard pituitary extract potentiated by cortisone is none of these hormones acting alone. The fact that more than one growth and gonadotrophic preparation was used strengthens this conclusion. None of the purified preparations contained appreciable thyroid-stimulating hormone, although there is little doubt that traces were present.

These hormones, ACTH plus perhaps other potentially active substances, are found in crude extracts of horse pituitary gland, which is very deficient in thyrotrophin. Such a preparation was very carefully assayed and 8.0 mg. found to contain but one thyroid-stimulating hormone unit as defined in the present study. Daily injection of eight mg. of the horse pituitary extract plus 12.5 mg. cortisone resulted in an excellent exophthalmos with the usual orbital modifications, save that the changes in the fat were relatively less striking than those of the Harderian gland.

Although the amount of thyroid-stimulating hormone administered daily was extremely little, both in the experiments with

the horse pituitary extract and in those using purified preparations, it was evident that a check was necessary on the possible action of small quantities in thyrotrophin when administered with cortisone. A relatively pure (equivalent in activity to the U.S.P. standard substance) thyrotrophin was carefully assayed and administered together with cortisone.

Three groups totaling 20 guinea pigs were given daily injection of 0.1 mg., 0.2 mg., or 0.4 mg. (2, 4, or 8 thyroid-stimulating hormone units) plus 5.0 to 12.5 mg. cortisone. Even the smallest quantity of this thyroid-stimulating hormone containing extract produced slight but unmistakable changes in the orbital fat and the protrusion of the globe. The surprising effectiveness of minute quantities of thyrotrophic extract, in the presence of cortisone, together with the absence of effect of the other recognized anterior pituitary factors (growth, prolactin, and gonadotrophin), lead one to believe that the exophthalmos and orbital fat changes at least are the result of thyroid-stimulating hormone plus adrenocorticoid action. However, the thyroid-stimulating hormone extract used was known to possess other physiologic activities.

In earlier experiments hypertrophy of both the orbital fat and Harderian gland always resulted from injection of pituitary extracts. In the present study there is some indication that these two tissues do not react to identical factors. These suggestive data are shown in Chart 4. The thyroid-stimulating hormone potency of all of the extracts administered is approximately the same, and in three instances the orbital fat hypertrophy was approximately 140 percent and that of the Harderian gland about 117 percent. In other experiments the fat hypertrophy was as great, or greater, but the Harderian gland response was essentially nil.

Although combinations of cortisone with either growth hormone, prolactin, or gonadotrophin failed to produce exophthalmos in two instances slight modification of the orbital fat was noted. Since the hormone preparations used were not pure, it is conceivable that they may have contained traces of some material with exophthalmogenic action or that they would have been effective if combined with other hormones. For these reasons combined injections of several of the purified fractions were made in an attempt to simulate the effect of the thyrotrophin-poor hormone mixture of the horse pituitary extract. Six guinea pigs were treated with a combination of growth hormone, prolactin, and cortisone containing less than 1.5 thyroid-stimulating hormone units as a contaminant. No exophthalmos nor hypertrophy of the Harderian gland resulted. There was, however, some change in the orbital fat which was suggestive of exophthalmos.

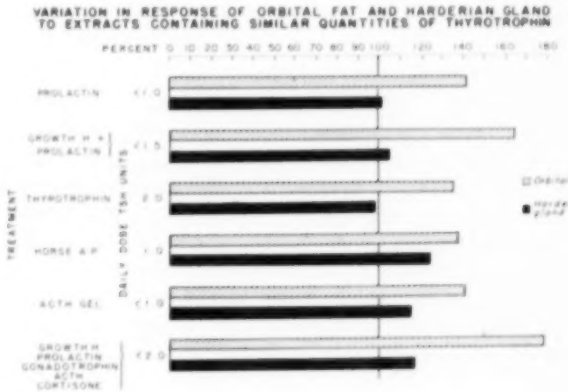


Chart 4 (Smelser and Ozanics). Variation in response of orbital fat and Harderian gland to extracts containing similar quantities of thyrotrophin.

A second experiment of this type was carried out on six guinea pigs in which all of the hormones save thyroid-stimulating hormone were combined. The quantities injected daily were growth hormone 2.5 mg., prolactin 1.0 mg., gonadotrophin 4.0 mg., ACTH 10 units, cortisone 5.0 mg., and thyroid-stimulating hormone, as a contaminant of these, less than two units. Definite exophthalmos was obtained in all cases (see chart) with marked edema and enlargement of the orbital fat, and a moderate hypertrophy of the Harderian gland.

The thyroid-stimulating hormone activity of the combined injections was determined both by totaling the quantities that had been found in each preparation when assayed separately, and also by subjecting test chicks to the same combination of injections which were given the guinea pigs. These tests were made at two-dose levels using 10 chicks per group, and included a U.S.P. thyrotrophic standard for comparison. The thyroid weight response to the combination was not greater than that given by two thyroid-stimulating hormone units of this standard.

Discussion

The experiments reported here confirm our earlier conclusion that at least two anterior pituitary factors are involved in the production of experimental exophthalmos. That ACTH, acting through the adrenal cortex, is one of these seems to be well established by the effectiveness of ACTH-B and of cortisone in potentiating exophthalmogenic extracts. The observation on the latter substance substantiates that of Aterman.[4]

Preliminary experiments with steroids other than cortisone have shown that this action is not limited to this particular steroid. Whether the ACTH and the adrenal cortex are requisite to, or merely accentuate, the exophthalmogenic process is not known.

Cortisone (32 mg./kg./day) does not produce exophthalmos in guinea pigs, as has been reported to occur in the rat.[5] However, both ACTH and cortisone exert some effect on the orbital tissues which may result from potentiation of endogenous pituitary factors. A slight increase in ocular protrusion, edema, and size of orbital fat result from thyroidectomy alone, which tends to modify the orbital tissues in the direction of exophthalmos. This process appears to be accentuated by ACTH or cortisone administration.

The mechanism by which adrenal steroids potentiate exophthalmos production by pituitary extract is not established. Some investigators have reported that cortisone administration provokes morphologic indication of thyroid hyperactivity,[6] although the reverse effect on thyroid function (I^{131} uptake) has been found by others.[7] D'Angelo[8] has suggested that cortisone augments the secretion of thyrotrophin by the hypophysis. If this should be true in exophthalmic animals, it contributes to our understanding of the mechanism of cortisone action in these cases and emphasizes the importance of the thyrotrophic hormone in the etiology of exophthalmos. On several occasions cortisone injection has been found to cause slight hypertrophy of the day old chick thyroid; however, it did not augment the response to small test doses of thyrotrophic hormone.

Although gonad hormones themselves may affect exophthalmos,[9] evidence is available showing that the gonadotrophins do not. Good exophthalmos has been produced with and without indication of gonadotrophic activity, and an active gonadotrophic preparation failed to produce an exophthalmos. The animals with good exophthalmos, produced by horse pituitary extract, had ovarian and uterine weights of 328 mg. and 945 mg. respectively (control value 48 mg. and 706 mg.). In contrast, guinea pigs with even better exophthalmos, caused by a purified thyroid-stimulating hormone extract, had ovarian and uterine weights of only 81 mg. and 431 mg. respectively. A purified horse

gonadotrophin failed to produce an exophthalmos, but the ovarian and uterine weights were 350 mg. and 1,298 mg.

No evidence was secured that prolactin played a rôle in any of the orbital changes proportional to its hormonal activity. Likewise, no indication could be found that growth hormone also has a rôle in exophthalmogenesis. This is of special interest in view of the many reports that exophthalmogenic extracts contain considerable quantities of the fat mobilizing principle.[10] Levin[11] has recently shown that the fat mobilizing factor is found in purified growth hormone extracts which are extremely active when administered in combination with cortisone. The failure of growth hormone and cortisone injections to cause exophthalmos in the present experiments casts strong doubt on the identity of the fat-mobilizing and exophthalmogenic factors.

Although very small quantities of thyrotrophic extracts produced exophthalmos and orbital fat modification, it is somewhat difficult to ascribe their effects entirely to the thyrotrophin they contain. The data presented here (see charts) show that appreciably greater skull-limbus measurements are obtained when the quantity of injected standard beef extract was increased two or four times. Therefore, some significance may be attached to differences in ocular protrusion on the order of 0.5 to 1.0 mm.

Administration of the combination of hormones included less than two thyroid-stimulating hormone units and resulted in an increase in the skull-limbus measurements of 0.8 mm. A total of eight thyroid-stimulating hormone units was required to produce a similar exophthalmos when the purified thyrotrophin was used. Only one thyroid-stimulating hormone unit of horse pituitary extract was required to duplicate the action of eight thyroid-stimulating hormone units of the purified preparation.

A similar situation exists with respect to the orbital fat which increased in weight appreciably as larger amounts of standard beef extract were given. Two and four thyroid-stimulating hormone units of the purified thyrotrophin caused orbital fat hypertrophy of 40 to 50 percent, whereas, a hypertrophy of 83 percent occurred when two thyroid-stimulating hormone units in the form of the combined hormones was administered. Eight thyroid-stimulating hormone units of the purified thyrotrophin were required to cause a comparable (77 percent) hypertrophy.

These discrepancies lead to the conclusion that some factor other than thyroid-stimulating hormone and the adrenal steroids is involved. Too much confidence, of course, should not be placed on differences which are small and variable. The biologic assay of pituitary hormones is subject to difficulties which render questionable claims of great accuracy; however, in this case we are dealing with a four-fold augmentation.

The finding of Dobyns and Steelman[12] that a thyrotrophin which was soluble in trichloracetic acid failed to produce exophthalmos in fish could have resulted from the separation of thyroid-stimulating hormone from the adrenal factor. Therefore, before thyroid-stimulating hormone may be completely exonerated an experiment with a pure thyrotrophin must be made.

The fact that cortisone augments exophthalmos caused by pituitary extracts is of significance in the understanding of its etiology. It has been suggested that exophthalmos results from an increase in the orbital connective tissue mast cells and the mucoids[13] which, it is postulated, is their product. Some investigators[14–16] find that cortisone markedly reduces the number of mast cells and the quantity of connective-tissue mucoid. If exophthalmos does result from an increase in the quantity of orbital connective tissue mast cells and mucoid ground substance, it is difficult to explain the striking augmentation of exophthalmos by cortisone, unless these reports are in error[17] or orbital

connective tissue is very different in its reaction from that in other parts of the body.

Summary

1. ACTH acting through the adrenal gland and adrenal steroids, exemplified by cortisone, augments the exophthalmos produced in guinea pigs by anterior pituitary extracts.

2. Neither ACTH nor cortisone in the quantities used in these experiments (32 mg. cortisone or 50 units ACTH/kg./day) causes appreciable proptosis, although some orbital changes are produced which are suggestive of minimal exophthalmos.

3. Exophthalmos is not produced through the interaction of cortisone with large quantites of either growth hormone, prolactin, or gonadotrophin.

4. The reported fat-mobilizing effect of growth hormone plus cortisone, and the failure of this hormonal combination to cause exophthalmos, strongly suggests that exophthalmogenic and fat-mobilizing factors are separate.

5. Exophthalmos, edema, and hypertrophy of the orbital fat are produced by concomitant administration of cortisone and small quantities of thyrotrophin in the form of either purified thyroid-stimulating hormone or crude horse pituitary extracts.

6. It is difficult to explain the exophthalmic changes solely on the basis of the quantity of thyroid-stimulating hormone administered, because combined injection of several purified anterior pituitary hormones containing only traces of thyroid-stimulating hormone are more effective than equal quantities of a purified thyrotrophin; therefore some additional hypothetical exophthalmogenic factor is postulated.

7. The strong exophthalmos-augmenting effect of cortisone requires a critical examination of the hypotheses:

a. That exophthalmos results from an increase in the quantity of mast cells and mucoid ground substance in the orbital connective tissue.

b. That cortisone markedly reduces the number of mast cells and mucoid ground substance of connective tissue.

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Discussion

Dr. Alson E. Braley: You are all well acquainted with the work Dr. Smelser has done on exophthalmos over a period of many years. It is excellent work.

Clinically I have been interested in this problem, as many of you know. There is very little doubt that something in the pituitary produces the exophthalmos, and I believe this factor is probably related to the ACTH; at least clinically in some instances you can produce some exophthalmos with ACTH.

I would like to issue a warning to those of you who use ACTH in quantities: You may stimulate an exophthalmos in a susceptible individual.

CHANGES IN THE PHOSPHATE FRACTIONS OF THE LENS UNDER VARIOUS CONDITIONS WHICH INFLUENCE CATION TRANSPORT*

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Previous reports from this laboratory[1,2] have discussed the relationship between hydration and cation distribution in the lens and have described a reversible lenticular cation shift useful in the study of lens metabolism. In particular, these studies have shown that the rabbit lens loses potassium and gains sodium during refrigeration at 0°C. During subsequent incubation at 37° C. in a proper environment, recovery toward the original cation composition occurs. Studies of the factors affecting this redistribution have indicated that the ability of the lens to reverse a cold-induced cation shift provides a sensitive index of lens viability.

The restoration of the cation distribution following refrigeration involves the movement of these ions against a concentration gradient and, therefore, requires energy. Studies of a variety of factors [including the effect of glucose and other monosaccharides, enzyme inhibitors, adenosine triphosphate (ATP), oxygen, and certain metabolites of the tricarboxylic acid cycle[3-6]], have indicated that the energy for active cation transport across lenticular barriers generally derives from the metabolism of carbohydrate.

Phosphorus plays an essential role in carbohydrate utilization. The present report concerns the effects of some conditions, known to alter cation transport, upon certain phosphate fractions [inorganic, easily hydrolyzable, and organic acid-soluble

* From the John E. Weeks Memorial Laboratory, Department of Ophthalmology, University of Oregon Medical School. Supported by Grant No. B-187 from the National Institutes of Health, National Institute of Neurologic Diseases and Blindness.

(OASP)] of the lens. Conditions were chosen for study which, as a group, provided a wide range of effects on cation recovery and phosphate fractions.

In particular, the answers to the following three questions were sought:

First, is there a causal relationship between cation transport and the concentration of the high energy phosphates, those compounds which supply the immediate energy for most cellular work?

Second, is cation movement related to the total quantity of organic acid-soluble phosphate in the lens?

Third, are the movements of inorganic phosphate and cations related?

A decrease in serum potassium and inorganic phosphate frequently occurs when carbohydrate utilization is stimulated in the intact animal.[6] The decrease in each instance is considered to result from the migration of the ions into the cell, the phosphate accumulating as organic esters.

Methods and Materials

To provide uniformity, lenses were handled in the same manner as in those experiments involving cation transport. Rabbit eyes were obtained from a rabbit slaughter house, the eyes being removed immediately after decapitation of the animal, dropped into normal saline at 0°C., and transported to the laboratory where the lens was extracted. Approximately one hour elapsed between enucleation and extraction. (The analyses listed in Table 1 as "Normal Fresh Lenses" are of lenses so treated. Control studies indicated that no significant change in the phosphorus fractions occurred during transportation to the laboratory.)

The lenses were then refrigerated at 0°C. for 41 to 45 hours in a modified Tyrode's solution under the conditions indicated.* At the end of this time, one of a pair of lenses from the same animal was analyzed immediately while the other was incubated for an additional six hours at 37°C. Additional details of the method have been previously described.[2-4]

For analysis, the lenses were rapidly weighed on a tissue balance and homogenized in ice-cold, six-percent trichloracetic acid. Inorganic phosphorus was determined by the method of Fiske and Subbarow.[7] Total acid-soluble phosphorus was measured as inorganic phosphorus determined after digestion of the filtrate with sulphuric acid and hydrogen peroxide. For the determination of the seven-minute-hydrolyzable phosphorus, a three ml. aliquot of the filtrate was heated with 0.8 ml. of 5N sulphuric acid in a boiling water bath for seven minutes prior to determination of inorganic phosphorus.

Results are calculated as milligrams of phosphorus per hundred grams of wet lens weight. As listed in the table, the seven-minute-hydrolyzable fraction consists of the phosphorus determined after heating as described, less the inorganic phosphorus determined immediately. The organic acid-soluble fraction consists of the total acid-soluble phosphorus minus the inorganic phosphorus. Each tabulated result represents the average of at least five individual analyses.

The phosphorus fractions here determined are essentially similar to those utilized by others.[8] The inorganic phosphorus is comprised of orthophosphate and phosphates

* We have published elsewhere the nature of the cation exchange during exposure to cold. During a period of 41 to 45 hours the potassium concentration of the lens drops from a normal level of 123.7 mEq. per 1,000 gm. of water to 75-85 mEq. per 1,000 gm. of water. The increase in sodium concentration is roughly equivalent but usually somewhat greater. After this period of refrigeration, recovery of the initial cation relationship during subsequent incubation at 37°C. in the basic medium is substantial but not complete. If refrigeration is more prolonged the lens gradually loses viability and becomes incapable of significant cation recovery. Recovery during incubation follows a first order curve and the new steady state is achieved by six hours. If incubation of refrigerated lenses is continued beyond this time, or if fresh lenses are incubated at 37°C., a potassium concentration greater than normal may be achieved.

split from very unstable esters such as creatine phosphate. Although including the phosphate from a variety of esters, the seven-minute-hydrolyzable fraction is considered a measure of such "high-energy" phosphates as adenosine triphosphate. For this study no attempt was made to determine or isolate the individual phosphate esters formed during the breakdown of glucose.

The determination of the cation recovery has been previously described.[2] The cation recovery data here included have been or will be reported elsewhere,[2-5] and are recorded here for convenience only.

Results

Effect of refrigeration

The phosphate fractions after refrigeration of the lens in various media are shown in Table 1. In general, those lenses that contained the least high-energy phosphate after refrigeration also showed the poorest recovery of cation content during incubation at 37°C., suggesting that the uptake of potassium depends at least in part upon the high-energy phosphate fraction.

Exceptions occurred, however, notably when the lens was massaged or bathed in media containing 0.001 M adenosine triphosphate or 0.001 M dinitrophenol. In these instances, a relatively high seven-minute-hydrolyzable phosphate level was observed, but the ability of the lens to concentrate potassium was limited. In general, cation transport during subsequent incubation at 37°C. was diminished if the initial level of easily hydrolyzable phosphate was low. On the other hand, a comparatively high concentration of this phosphate fraction did not guarantee good cation recovery.

The concentration of organic acid-soluble phosphorus observed after refrigeration was found to vary quite markedly with the procedure. No exact correlation between the level of organic acid-soluble phosphorus and the migration of potassium against a concentration gradient during subsequent incubation at 37°C. was observed. However, where the concentration of organic-acid soluble phosphorus was low following refrigeration, the ability of the lens to concentrate cations during subsequent incubation at 37°C. was limited. On the other hand, poor recovery was often observed when the

TABLE 1

PHOSPHATE FRACTIONS OF THE RABBIT LENS DETERMINED AFTER REFRIGERATION AT 0°C. FOR 41–45 HOURS IN MODIFIED TYRODE'S SOLUTION WITH VARIOUS ADDITIVES

Media Additive or Condition Imposed	Phosphorus—mg./100 gm. Wet Weight				Potassium Recovery—Percent*
	Total Acid Soluble	Inorganic	Organic Acid-Soluble	Seven-Minute-Hydrolyzable	
Normal Fresh Lens	56.2	9.3	46.9	15.7	—
1. None	57.1	5.2	51.9	15.2	78
2. Massage	74.3	7.8	66.5	15.9	13
3. Anaerobic atmosphere	40.8	3.3	37.5	10.6	13
4. Absence of glucose	51.3	14.7	36.6	8.9	0
5. 0.01M pyruvate, no glucose	57.3	8.4	48.9	12.9	0
6. 200 mg. % fructose, no glucose	48.5	5.7	42.8	10.5	30
7. 500 mg. % fructose, no glucose	53.6	9.6	44.0	14.1	84
8. 200 mg. % galactose, no glucose	54.0	13.7	40.3	11.2	9
9. 0.001M adenosine triphosphate	54.1	2.1	52.0	14.5	36
10. 500 mg. % glucose and 0.001M adenosine triphosphate	68.3	4.3	64.0	10.9	78
11. 0.001M NaCN	71.4	14.2	57.2	6.6	0
12. 0.025M iodoacetic acid	41.3	8.6	32.7	5.8	0
13. 0.001M dinitrophenol	66.2	5.9	60.3	16.5	12

* Data here presented have been published elsewhere (see text).

organic acid-soluble phosphorus value was relatively high.

While utilization of glucose by the lens is virtually nil at 0°C., it is interesting to note that metabolic activity is far from suspended at this temperature. Thus, marked changes in phosphate fractions do occur and, while these changes do not correlate exactly with the cation movement, it is obvious that lenses which have been refrigerated under various conditions are not capable of the same response to the stimulus that subsequent incubation at 37°C. provides.

Effect of refrigeration and subsequent incubation at 37°C.

The phosphate fractions obtained after refrigeration and subsequent incubation are listed in Table 2. Again, the active transport of cations was not likely to occur in those instances where the final values of high-energy phosphate and organic acid-soluble phosphorus were low. On the other hand, a comparatively high value of either did not insure movement of cations against a concentration gradient.

The changes in phosphate fractions which occurred during incubation at 37°C. are listed in Table 3. It will be recalled that the movement of cations against a concentration gradient may occur during this period. No correlation between the changes in the organic acid-soluble phosphorus or seven-minute hydrolyzable fractions and active transport of cations was observed. If present, a relationship between the movement of inorganic phosphate and potassium should be most apparent during the recovery phase; that is, during incubation at 37°C. following refrigeration.

The change in inorganic phosphorus concentration in the lens does not of necessity reflect the exchange of phosphate across the lens barriers since hydrolysis or synthesis of organic phosphate may alter the level. However, changes in the total acid-soluble phosphorus probably provide a fair index of movement of inorganic phosphate, assuming that the organic esters do not diffuse across the barrier.

Using this index, no relationship was observed between cation movement and the movement of inorganic phosphorus. In certain instances, for example, in the presence

TABLE 2

PHOSPHATE FRACTIONS OF THE RABBIT LENS DETERMINED AFTER REFRIGERATION AT 0°C. FOR 41–45 HOURS AND SUBSEQUENT INCUBATION AT 37°C. FOR SIX HOURS IN MODIFIED TYRODE'S SOLUTION WITH VARIOUS ADDITIVES

Media Additive or Condition Imposed	Phosphorus—mg./100 gm. wet weight				Potassium Recovery—Percent*
	Total Acid Soluble	Inorganic	Organic Acid-Soluble	Seven-Minute-Hydrolyzable	
1. None	51.7	7.7	44.0	11.9	78
2. Massage	62.4	10.9	51.5	9.1	13
3. Anaerobic atmosphere	38.1	8.6	29.5	6.0	13
4. Absence of glucose	53.7	24.1	29.6	4.1	0
5. 0.01M pyruvate, no glucose	51.4	18.3	33.1	7.1	0
6. 200 mg. % fructose, no glucose	45.9	18.4	27.5	2.4	30
7. 500 mg. % fructose, no glucose	55.5	15.6	39.9	10.4	84
8. 200 mg. % galactose, no glucose	65.9	15.9	50.0	11.2	9
9. 0.001M adenosine triphosphate	53.3	11.0	42.3	10.5	36
10. 500 mg. % Glucose and 0.001M adenosine triphosphate	46.7	7.2	39.5	7.6	78
11. 0.001M NaCN	80.0	13.6	66.4	7.7	0
12. 0.025M Iodoacetic acid	50.5	8.1	42.4	1.0	0
13. 0.001M dinitrophenol	68.9	16.5	52.4	11.0	12

* Data here presented have been published elsewhere (see text).

TABLE 3

CHANGES IN THE PHOSPHATE COMPOSITION OF THE RABBIT LENS WHICH OCCUR DURING INCUBATION AT 37°C. FOR SIX HOURS IN MODIFIED TYRODE'S SOLUTION WITH VARIOUS ADDITIVES, SUBSEQUENT TO REFRIGERATION AT 0°C. FOR 41–45 HOURS

(The values were obtained by subtracting the appropriate data in Table 2 from that in Table 1)

Media Additive or Condition Imposed	Phosphorus—mg./100 gm. Wet Weight				Potassium Recovery—Percent*
	Total Acid Soluble	Inorganic	Organic Acid-Soluble	Seven-Minute-Hydrolyzable	
1. None	− 5.4	+ 2.5	− 7.9	−3.3	78
2. Massage	−11.9	+ 3.1	−15.0	−6.8	13
3. Anaerobic atmosphere	− 2.7	+ 5.3	− 8.0	−4.6	13
4. Absence of glucose	+ 2.4	+ 9.4	− 7.0	−4.8	0
5. 0.01M pyruvate, no glucose	− 5.9	+ 9.9	−15.8	−5.8	0
6. 200 mg. % fructose, no glucose	− 2.6	+12.7	−15.3	−8.1	30
7. 500 mg. % fructose, no glucose	+ 1.9	+ 6.0	− 4.1	−3.7	84
8. 200 mg. % galactose, no glucose	+11.9	+ 2.2	+ 9.7	0	9
9. 0.001M adenosine triphosphate	− 0.8	+ 8.9	− 9.7	−4.0	36
10. 500 mg. % glucose and 0.001M adenosine triphosphate	−21.6	+ 2.9	−24.5	−3.3	78
11. 0.001M NaCN	+ 8.6	− 0.6	+ 9.2	+1.1	0
12. 0.025M iodoacetic acid	+ 9.2	− 0.5	+ 9.7	−4.8	0
13. 0.001M dinitrophenol	+ 2.7	+10.6	− 7.9	−5.5	12

* Data here presented have been published elsewhere (see text).

of cyanide, a movement of inorganic phosphorus into the lens against a concentration gradient must have occurred, yet cation recovery was nil.

COMMENT

The failure to demonstrate an exact correlation between the concentration of organic acid-soluble phosphorus and cation recovery indicates that the physical-chemical properties of these substances (nondiffusible anions) have little influence in cation distribution. It is not surprising that cation recovery is minimal or abolished when the concentration of organic acid-soluble phosphorus or seven-minute-hydrolyzable phosphate is low. Of the two fractions the easily hydrolyzable phosphate best correlates with active transport of cations. The observation that active transport may not occur even when the concentration of the seven-minute hydrolyzable fraction is relatively high may simply reflect the fact that cation distribution is a balance of many factors. Moreover, a static analysis provides no information concerning the rate of turnover of the phosphate esters.

SUMMARY

No exact correlation was observed between cation transport and the concentration of organic acid-soluble phosphorus or seven-minute-hydrolyzable phosphorus of the lens. No parallel between the movement of cation against a concentration gradient and the movement of inorganic phosphorus was observed.

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Discussion

Dr. A. M. Potts: I would like to ask Dr. Hauschildt in particular whether the rate of inhibition as glucose concentration goes up past 200 mg. percent drops off linearly or whether it drops off suddenly.

As Dr. Patterson at Western Reserve has shown, there apparently is a direct connection between glucose level at the lens and cataractogenesis in diabetes. It is hard to conceive that glucose concentration in a diabetic will remain significantly higher than 200 or 250 mg. percent over extended periods. If his concentrations become suddenly inhibitory above 200 mg. percent, it might be quite conceivable that this is a mechanism connected with diabetic cataract. If levels of 500 mg. percent are required for effect, it is a little difficult to conceive a physiologic role for this mechanism.

I also would like to ask Dr. Harris whether, in the concentrations in which these inhibitors were used, and in consideration of the partial inhibition of glucose absorption, there is room for a considerable amount of glucose absorption by diffusion as well as by metabolic transfer.

Dr. James H. Hauschildt: I will answer Dr. Pott's questions first.

Does the rate of inhibition go up linearly? I can't give you as complete an answer as I would like to. We have not tested with phosphate measurements any concentrations between 200 and 500 mg. percent of glucose. However, we have checked these different concentrations of glucose with respect to potassium and sodium recovery, and I can answer that the inhibitory effect is not linear.

To elaborate a bit, we have investigated glucose by itself up to as high as 600 mg. percent, and to rule out osmotic effects we also have added sucrose to make the total osmotic pressure as much as one would get with 600 mg. percent of glucose.

The sucrose itself has no marked effect. That would argue a bit against a strictly physical-chemical effect. In other words, we are not observing a mass-action phenomenon. I can't fully answer the question about the utilization of glucose as it is influenced by concentration. I will refer that question to Dr. Harris.

With respect to metabolic inhibitors such as iodoacetate—we recognize 0.01 molar as a very high concentration. We had no particular reason for picking it except that for about three years or so we have been using 0.01 molar metabolites such as members of the Krebs cycle, and other additives to the medium have been about 0.01 molar. This is, perhaps, poor justification for using iodoacetate at this concentration. I have an idea that the effects would be noticeable in much lower concentrations, because it is a rather specific enzyme poison.

Dr. John E. Harris (closing): We have been rather intrigued by the high level of glucose that we get in our diabetic lenses, Dr. Potts. We have done considerable work on that, which we did not report here because of time limitations, with the idea that generally one can demonstrate in vitro effects of insulin only in diabetic tissues.

Our technique failed when we tried to use diabetic lenses. We took the lenses at the end of 60 hours (60 hours after the injection of alloxan), when the blood sugars ran from 350 to 600 mg. percent. At the end of that time we found a considerable concentration of glucose in the lens, much higher than 200 mg. percent. Generally it ran in the neighborhood of 300 to 400 mg. percent. Therefore, our technique was not reliable.

Interestingly enough, under those circumstances we actually found a depletion of glucose when insulin was added, suggesting that in diabetic tissues we may have an insulin effect that is not possible to demonstrate in normal tissues. The problem is being studied using Carbon-14 labeled glucose.

I might add that we have used a wide range of concentrations of iodoacetic acid in our work on cation transport. We used it in considerably lower and somewhat in higher concentrations and, as a matter of fact, we did find that in lower concentrations we get an inhibition of the cation transport. This happened to hit a relatively median value at the time of our experiments.

The question of glucose utilization and the effect of concentration on it has not been specifically examined in the lens to my knowledge. It has been examined by a number of experimentalists in other tissues, such as Wick and Drury, as well as others. Using concentrations up to 1,000 mg. percent and generally using the intact but eviscerated animal, they did find a steady increase in glucose utilization as the concentration was increased under the conditions of their experiment.

We have not studied that problem with the lens since the utilization of glucose is too slight to be measured with accuracy when concentrations of 500 to 1,000 mg. percent are employed.

TRANSPORT OF GLUCOSE ACROSS THE LENS SURFACES*

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AND LORETTA T. NORDQUIST, B.A.
Portland, Oregon

It has been observed that glucose in sufficiently high concentration (500 mg. percent or more) has a deleterious effect on the transport of cations by the lens, and that adenosine triphosphate, which provides the immediate energy for the phosphorylation by which glucose enters the glycolytic cycle, tends to negate these adverse effects.[1]

These observations suggested that glucose might be metabolically active at some lens surface, and, furthermore, that the movement of glucose into the lens from the extralenticular medium might be metabolically mediated. Such a concept has, indeed, been advanced by Müller, who observed that the glucose accumulated by the lens was decreased in the presence of iodoacetic acid.[2] The problem is important since the cause of various cataracts is often considered to be diminished supply to the lens of nutrient material, which normally consists mainly of glucose.

Our interest in the problem was heightened by the prospect that study of the lens might contribute to a better understanding of the mechanism of glucose transport across cellular membranes in general. Unlike many cells, the lens normally contains an appreciable quantity of free glucose; hence measurement of its accumulation is feasible. In addition, the lens is an avascular structure which can be removed in its entirety for in vitro study with little disturbance to its nutrient supply. For these reasons the lens was considered to be an excellent tissue for studies aimed at determining whether glucose crosses cellular barriers by simple diffusion or metabolic mediation or both.

Two reasonable approaches to the problem present themselves. The first involves measurement of glucose accumulation under various conditions in the lens incubated in media containing a relatively high glucose concentration, concomitant with studies of depletion of glucose from the medium. The second approach consists of measurements of the turnover of C^{14}-labelled glucose. The present communication is concerned with the first approach.

METHODS

Rabbit lenses were used throughout. These were removed by a posterior approach in a manner previously described.[3] Generally, two lenses from different animals were incubated at 37°C. in a small Erlenmeyer flask containing 10 ml. per lens of a modified Tyrode's solution[3] to which extra glucose was added.

The media was equilibrated with a 95 percent O_2-5.0 percent CO_2 mixture, except when anaerobic conditions were used. Unless otherwise specified the glucose concentration employed was 400 mg. percent and the period of incubation six hours. Under these circumstances the glucose concentration of the bathing medium remained substantially constant under all experimental conditions.

At the end of the period of observation the lenses were rapidly weighed, the two of them homogenized together and the proteins preciptated with barium hydroxide and zinc sulphate. Reducing substance was determined using the method of Somogyi[4] both before and after fermentation with yeast.

Results are expressed as apparent glucose, which includes both the fermentable and

* From the John E. Weeks Memorial Laboratory, Department of Ophthalmology, University of Oregon Medical School. Supported by a grant from the Diabetic Research Foundation of Portland, Oregon.

nonfermentable reducing substance, or as glucose, which is the fermentable reducing substance only.

Employing the experimental procedure and analytic technique here described, there is little doubt that the fermentable reducing substance includes only free glucose. Such other reducing and fermentable substances as glucose-6-phosphate are precipitated by the barium hydroxide-zinc sulfate mixture.

Concentrations are usually computed in units of wet lens weight, since the water content of the lens could not be determined under the conditions of this experiment. In specified instances results are expressed as concentration per unit of water, using a previously determined average water content for normal lenses of the size employed (66 percent[5]). With rare exception, each bar or point in the figures represents the average of at least eight analyses of as many pairs of lenses.

In addition, the quantity of glucose lost from the bathing medium to the lens was determined under many of the conditions employed. For this purpose lenses were incubated singly for six hours in three ml. of a modified Tyrode's solution (200 mg. percent glucose) and the change in glucose concentration of the bathing medium noted. Results are computed in terms of mg./hour/gm. of wet lens weight. Under the conditions of this experiment the figures obtained provide a measure largely of the glycolytic activity of the lens. Except when the glycolytic poisons were used, the accumulation of glucose within the lens accounted for only approximately one tenth of the total glucose lost from the medium.

Normal glucose levels of the lens

Thirty-six analyses of fresh lenses demonstrated an average total reducing substance equivalent to 43.4 mg. of glucose per 100 gm. of wet lens. Of this apparent glucose an average of 23.6 mg. was found to be nonfermentable reducing substance. Thus, by difference, the true glucose level averaged 19.8 mg. per 100 gm. of wet lens. This is lower than values reported by others,[6] a discrepancy which is probably accounted for by the difference in analytic techniques. The quantity of nonfermentable reducing substance was quite constant under the various experimental situations and did not differ significantly from that found in fresh lenses.

Kinetics of glucose uptake by the lens

The first step in the analysis of this problem was to determine whether the accumulation of glucose within the lens was proportional to its concentration in the bathing medium. The existence of such a proportionality would not necessarily distinguish between an active and a passive process, but lack of such a proportionality would rule out a movement by simple diffusion. Two approaches were utilized:

The first was the measurement of the change in concentration of lens glucose with time. Lenses were incubated in modified Tyrode's solution (containing 200 mg. percent glucose) and analyzed for glucose content after various time intervals. The results did not fall on the simple exponential curve to be expected if the movement were one of simple diffusion (fig. 1). This deviation may be due to failure of the entering glucose to distribute itself equally throughout the lens substance.* The results therefore are not incompatible with the view that the accumulation of glucose within the lens is proportional to the glucose concentration in the bathing medium.

The second approach was to measure glucose accumulation by lenses incubated for a constant time interval (three hours) in

* Another source of discrepancy was a decrease in glucose concentration of the bathing medium which became significant after 10 hours' incubation. This error is minimized by plotting the ratio of lens to media glucose rather than the concentration in the lens as the ordinate.

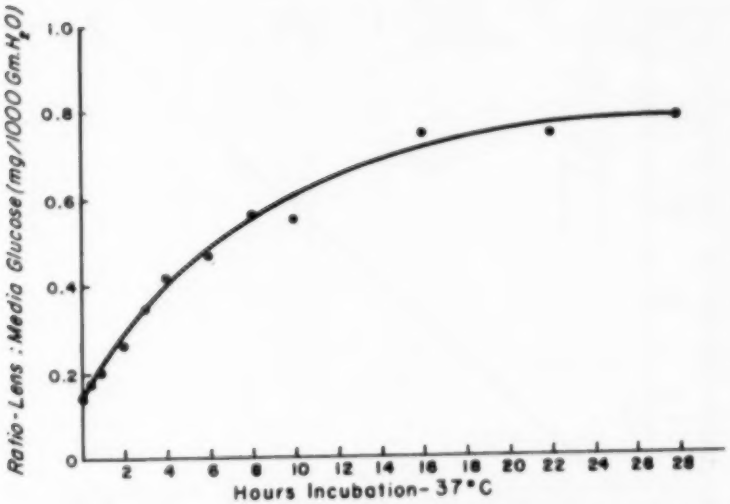

Fig. 1 (Harris, Hauschildt, and Nordquist). Ratio of lens : media glucose after incubation of rabbit lenses at 37°C. for various time intervals in modified Tyrode's solution containing 200 mg. percent glucose. Each point represents at least eight analyses, except the 22- and 28-hour points, which represent six and four analyses, respectively.

media containing various glucose concentrations. The glucose content of the lens was found to be a linear function of the concentration of the external medium (fig. 2), indicating that the accumulation of glucose within the lens is a simple function of the concentration presented to it. Such results are compatible with the view that glucose passively diffuses into the lens although the possibility of a metabolic mediation, also proportional to the concentration, cannot be excluded.

Obviously, the measured accumulation after a given time interval represents a balance between the amount of glucose which enters the lens and that utilized by it. Interpolation from the graph (fig. 2) indicates that that concentration in the external medium which will just maintain the normal lens glucose during a three hour period is 122 mg. percent. This is somewhat higher than the values usually reported for aqueous glucose concentration. The reason for this discrepancy is not immediately apparent.

Effect of injury to the lens and its capsule

Studies of active transport of cations have indicated that incision of the lens capsule or seemingly minor injury (massage) of the lens surface markedly alters its ability to concentrate the positive ions.[7] The effect of such manipulations on the ability of the lens to accumulate glucose was accordingly investigated.

The lens capsules were incised on either their anterior or posterior surface, the incision extending approximately two thirds of the lens diameter, and a lip of capsule remaining inside the equator at either end of the incision. Lenses of another group were gently massaged on one or the other surface with a blunt glass rod.

The accumulation of glucose by both groups was compared with that of uninjured lenses. Neither incision nor massage of the capsule significantly altered the rate of accumulation of glucose by the lens (fig. 3). The uptake of glucose from the media and

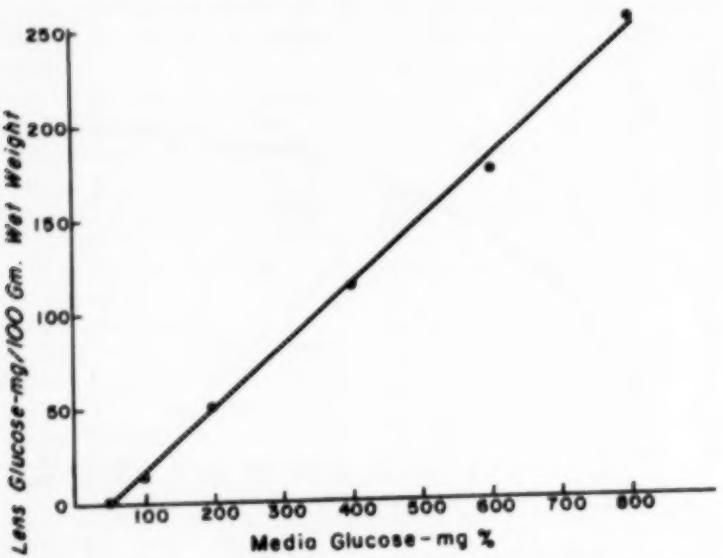

Fig. 2 (Harris, Hauschildt, and Nordquist). Glucose concentration of rabbit lenses after incubation at 37°C. for three hours in modified Tyrode's solution containing various glucose concentrations. Each point represents at least eight analyses.

hence the utilization of glucose was significantly reduced when the capsule was incised (table 1).*

Effect of anaerobic conditions and various metabolic poisons

Lenses were incubated as previously described, but in a medium equilibrated with a 95 percent N_2-5.0 percent CO_2 mixture. As compared with lenses respiring normally but otherwise under identical circumstances, the accumulation of glucose within the lens was reduced in the absence of oxygen (fig. 4).

In the presence of cyanide (and oxygen) the accumulation of glucose was diminished even more. Cyanide has been shown to inhibit respiration of tissues, including the lens.[8] However, the anaerobic breakdown of glucose can proceed in either the presence of cyanide or the absence of oxygen. The fact that the depletion of glucose from the bathing media was increased under anaerobic conditions or in the presence of cyanide (table 1) supports the view that the lowered

TABLE 1

Depletion of media glucose by the lens incubated at 37°C. for six hours in modified Tyrode's solution with various additives

Media Additive	Glucose Depletion (mg./hr./gm. of wet lens wt.)
None	0.78
None, incised capsule	0.65
Anaerobic	1.09
0.001 M NaCN	1.18
0.01 M dinitrophenol	0.62
0.025 M iodoacetic acid	0.13
0.05 M NaF	0.30
0.05 M NaF, incised capsule	0.28
0.1 unit insulin/ml.	0.87
0.1 unit insulin/ml., incised capsule	0.79

* Here, as elsewhere in the paper, the question of the significance of differences between means has been resolved by employing the t-test, accepting $p = 0.05$ as the confidence level.

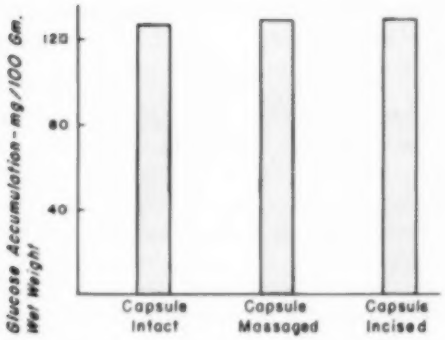

Fig. 3 (Harris, Hauschildt, and Nordquist). Effect of injury to the lens capsule on the accumulation of glucose in rabbit lenses incubated at 37°C. for six hours in modified Tyrode's solution containing 400 mg. percent glucose.

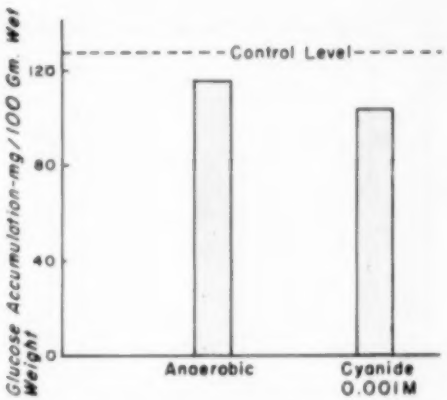

Fig. 4 (Harris, Hauschildt and Nordquist). Effect of cyanide and anaerobic conditions on the accumulation of glucose in rabbit lenses incubated at 37°C. for six hours in modified Tyrode's solution containing 400 mg. percent glucose. Control level is accumulation of glucose through the intact capsule as given in Figure 3.

accumulation in these cases is probably due to increased glucose utilization rather than to decreased glucose transport.

To negate the influence of utilization on the measured accumulation, substances which inhibit glucose breakdown were employed. For these studies dinitrophenol, sodium fluoride, and iodoacetic acid were chosen. In the presence of any of these the depletion of glucose from the medium was found to be reduced (table 1).

Dinitrophenol is an uncoupling agent which by inhibiting oxidative phosphorylation depletes the supply of high energy phosphates. In low concentrations it stimulates oxygen consumption of the lens[8] and other tissues,[10] but depresses respiration and glucose utilization in higher concentrations.

In appropriate concentration (0.01 M) dinitrophenol markedly inhibited glucose accumulation by the lens (fig. 5). In the presence of either sodium fluoride or iodoacetic acid, both of which inhibit specific reactions in the glycolytic sequence, the accumulation of glucose by the lens was similarly reduced (fig. 6). The results indicate that these substances inhibit the transport of glucose into the lens.

The possibility that the lowered glucose uptake by lenses incubated with dinitrophenol, fluoride, or iodoacetate resulted from a decrease in available adenosine triphosphate or a decreased turnover of adenosine triphosphate was next considered. (A decreased turnover of adenosine triphosphate in erythrocytes poisoned with iodoacetic acid or sodium fluoride has been re-

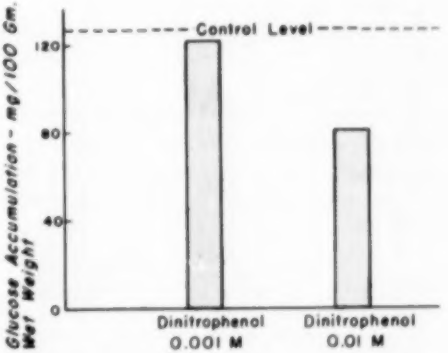

Fig. 5 (Harris, Hauschildt, and Nordquist). Effect of dinitrophenol on accumulation of glucose in rabbit lenses incubated at 37°C. for six hours in modified Tyrode's solution containing 400 mg. percent glucose. Control level is accumulation of glucose through the intact capsule as given in Figure 3.

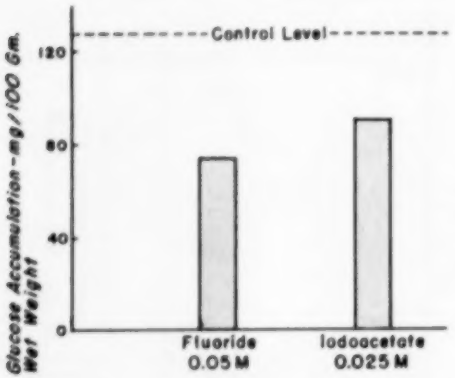

Fig. 6 (Harris, Hauschildt, and Nordquist). Effect of iodoacetate and fluoride on accumulation of glucose in rabbit lenses incubated at 37°C. for six hours in modified Tyrode's solution containing 400 mg. percent glucose. Control level is accumulation of glucose through the intact capsule as given in Figure 3.

ported.[11]) To test this possibility the effect of adding both adenosine triphosphate (0.001 M) and an enzyme inhibitor (fluoride) was observed. In the presence of adenosine triphosphate the accumulation of glucose was significantly increased and hence the inhibition induced by fluoride at least partially overcome (fig. 7, table 1).

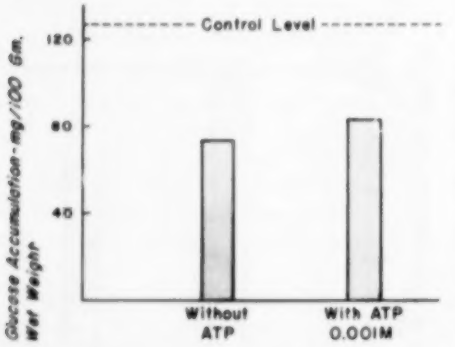

Fig. 7 (Harris, Hauschildt, and Nordquist). Effect of added adenosine triphosphate (ATP) on accumulation of glucose in rabbit lenses incubated at 37°C. for six hours in modified Tyrode's solution containing 400 mg. percent glucose and 0.05 M sodium fluoride. Control level is accumulation of glucose through the intact capsule as given in Figure 3.

Effect of insulin and adenosine triphosphate

One of the postulated effects of insulin is the promotion of glucose transport across cellular barriers.[12] The effect of insulin on glucose accumulation by the lens was accordingly studied. In a concentration of 0.1 unit per ml. of media, insulin did not significantly affect the accumulation of glucose by the lens (fig. 8). In the experimental series here reported glucose utilization by the lens with an intact capsule was not significantly altered by insulin (table 1).

Insulin activity in vitro can generally be most adequately demonstrated in diabetic tissues. (A valid question arises whether insulin is normally present in the aqueous humor. Studies in our laboratory using as a tracer insulin iodinated with radioactive iodine have at present not conclusively ruled out the possibility that some insulin enters the aqueous.)

A study of the effect of insulin upon glucose accumulation by the lenses of alloxan-diabetic rabbits was attempted but the described procedure was not found to adapt well to this technique because of the

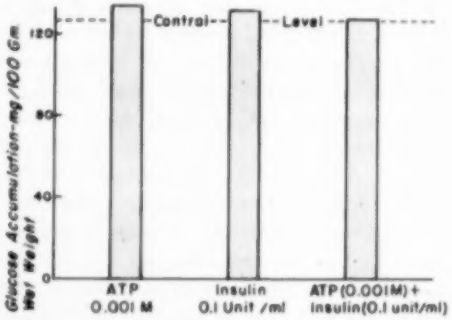

Fig. 8 (Harris, Hauschildt, and Nordquist). Effect of adenosine triphosphate, insulin, and adenosine triphosphate, plus insulin on accumulation of glucose in rabbit lenses incubated at 37°C. for six hours in modified Tyrode's solution containing 400 mg. percent glucose. Control level is accumulation of glucose through the intact capsule as given in Figure 3.

initially high and variable glucose levels in the diabetic lenses.

It is of interest, however, that when the two lenses from a diabetic animal were incubated, one with and one without added insulin in media containing 400 mg. percent glucose, the total lens glucose after six hours was in every instance lower in the lens incubated with insulin than in the one where the hormone was not employed. This may indicate that glucose utilization by the diabetic lens was increased when insulin was added. However, an unequivocal determination of the effect of insulin on the transport and utilization of glucose by the intact lens must await the results of studies with C^{14}-labelled glucose.

The effect of adenosine triphosphate, both with and without added insulin, was also studied, using normal rabbit lenses. Adenosine triphosphate did not significantly alter the accumulation of glucose by the lens (fig. 8), perhaps indicating that adenosine triphosphate is normally present in the lens in optimal concentration; hence an added quantity does not significantly alter the rate of glucose uptake.

Site of the barrier to glucose

Our experiments with cations have indicated that the barrier across which cation transport occurs lies at or in close proximity to the lens capsule. Whether a similar barrier is involved in the transport of glucose was tested by comparing glucose accumulation with the capsule intact and incised. The accumulation in the two preparations was found not to differ significantly (fig. 1).

(One might reason that since the utilization was reduced when the capsule was incised, the overall transport into the lens was greater through the intact capsule and that, therefore, some active mediation must occur at the capsule. Such a conclusion from this data, however, would be hazardous.) However, the employment of an enzyme inhibitor known to reduce glucose transport provides a better test for such a barrier. Thus, in media containing fluoride a greater accumulation of glucose was observed in the lens with an incised capsule than in the one with the capsule intact (fig. 9, table 1).

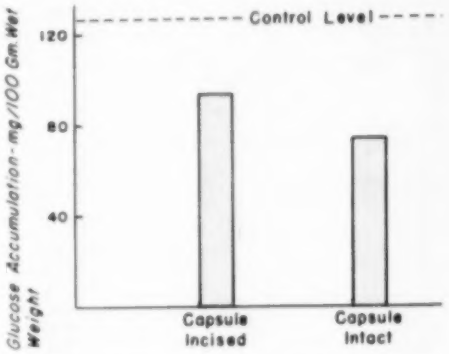

Fig. 9 (Harris, Hauschildt, and Nordquist). Effect of incision of the capsule on accumulation of glucose in rabbit lenses incubated at 37°C. for six hours in modified Tyrode's solution containing 400 mg. percent glucose and 0.05 M sodium fluoride. Control level is accumulation of glucose through the intact capsule as given in Figure 3.

The results support the view that the glucose transport mechanism acts at some barrier at or near the lens capsule. They do not rule out the possible existence of some other barrier to movement which is poisoned by fluoride.

When the lens with incised capsule was incubated in the presence of insulin, the glucose accumulation was significantly less than when either the intact lens was incubated with insulin, or the lens with incised capsule was incubated without insulin (fig. 10; compare fig. 3).

At the same time the depletion of glucose from the bathing medium by lenses with incised capsules was significantly greater when insulin was present (table 1). These data are compatible with those of Ross.[13] However, it is reasonable to take issue with his conclusion that the stimulating effect of insulin on the uptake of glucose by decapsu-

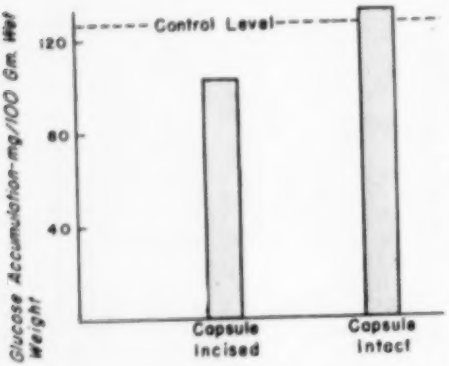

Fig. 10 (Harris, Hauschildt, and Nordquist). Effect of incision of the capsule on accumulation of glucose in rabbit lenses incubated at 37°C. for six hours in modified Tyrode's solution containing 400 mg. percent glucose and 0.1 unit insulin per ml. Control level is accumulation of glucose through the intact capsule as given in Figure 3.

lated lens substance is due to the mediation of glucose transport across a cellular barrier. From present evidence, one can only conclude that insulin stimulates glucose utilization by this tissue in a manner which remains to be clarified.

Comment

The results leave little doubt that the transport of glucose into the lens is metabolically mediated and that the movement is proportional to the concentration in the external medium. Since in no instance was a higher concentration of glucose achieved in the lens than was present in the surrounding fluid, this transport cannot be considered an active transfer. Rather the term "assisted transport" might be preferable. No specific mechanism such as phosphorylation of glucose can be implied from our data.

The possibility certainly exists that a highly dissociable complex of glucose with some unknown substance is formed at the limiting surfaces. Such a complex has been postulated to mediate the movement of glucose into the erythrocyte.[14] However, some relation of glucose transport to the high energy phosphates seems likely.

The hypothesis that inorganic phosphate enters the erythrocyte by forming adenosine triphosphate, that this process is poisoned by fluoride and iodoacetate ions, and that it is closely associated with glycolysis is of interest.[11,15] The movement of phosphate and glucose may be closely associated. It is emphasized, however, that our results do not rule out the possibility that some glucose enters the lens by simple diffusion.

The question of the site of this transport cannot be finally answered. Certainly, at least one site lies at or near the lens capsule. The sum total of our experience with cation and glucose transport has strengthened the concept that the lens acts much as a single large cell, whose limiting barrier is near the lens surface and which shows the compartmentalization of metabolic activity typical of a single cell. Much clinical experience, such as the cataractogenic effect of trauma, is best interpreted from this viewpoint.

The contrast of the data here presented with those obtained under similar circumstances but concerning lens cation balance is sufficiently striking to deserve comment. For example, when the lens is poisoned with sodium fluoride or iodoacetic acid a cation shift toward equilibrium with the external medium is observed.[5] In respect to glucose, however, these poisons reduce the tendency toward equilibrium with the external medium. This contrast emphasizes that the results in each instance are not due to a nonspecific effect on permeability of the barriers but rather to the inhibition of some more specific metabolic process or processes.

Summary

1. Studies of the accumulation of glucose within and the utilization of glucose by the rabbit lens treated with various enzyme inhibitors have indicated that glucose does not enter the lens solely by simple diffusion but also by some active process involving metabolic mediation.

2. One site or barrier at which this process occurs lies at or in close proximity to

the lens capsule. Some other barrier may also exist.

3. The role of insulin in the transport of glucose across the lens barriers has not been established.

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A SCINTILLATION COUNTER FOR THE DIAGNOSIS OF INTRAOCULAR MELANOMAS*

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San Francisco, California

Eyes containing lesions which simulate melanomas are sometimes enucleated unnecessarily. At times the removal of intraocular melanomas is unfortunately delayed because of an error in diagnosis. An instrument which would definitely aid in the differential diagnosis of melanomas and benign intraocular lesions is highly desirable.

Radioactive phosphorus (P^{32}) given intravenously in the form of sodium biphosphate is taken up in higher concentrations by actively growing tumor tissue than by benign tissue. This fact was first demonstrated clinically when P^{32} was used as a diagnostic aid in the detection of breast tumors by Low-Beer in 1946.[1] It was originally applied to ophthalmology in the differential diagnosis of intraocular lesions by Thomas, Krohmer, and Storaasli in 1952.[2]

In most organs other than the eye a biopsy is easier and more accurate than the determination of activity over a lesion after the injection of a radioactive substance. For this reason, the use of P^{32} or other radio-isotopes has not found wide acceptance as a substitute for biopsy. In ophthalmology, however, the situation is different. Biopsies of intraocular tumors are generally unsatisfactory and sometimes dangerous or difficult. For this reason, the possibility of arriving at a more accurate differential diagnosis of malignant and nonmalignant intraocular lesions by the use of radio-isotopes cannot be put aside lightly.

If the differential diagnosis of malignant intraocular tumors through the use of radioactive phosphorus is to be reasonably accurate, two essential steps must be followed:

1. The counts of radioactivity must be made a long while after the injection of P^{32}.

2. The counter must be placed directly over the lesion in the closest possible proximity.

If these two steps are not followed the test is grossly inaccurate and very misleading.[3] Therefore, these steps must be considered in more detail.

There is reason to believe that more accurate differential counts will be obtained 24 hours or more after the injection of P^{32}. During the first 15 minutes after injection, the phosphorus is largely free in the blood stream.[4] During the remainder of the first hour it is diffusing into the tissues. Counts taken at this time only indicate the vascularity of the tissue, rather than the rate of cellular growth.

After phosphorus has diffused into the tissues it is found combined with carbohydrates, fats, and proteins. In general, in most tissues studied, the combination of radioactive phosphorus is evident first in the carbohydrate, second in the fat portion, and third in the protein.[5] The phosphorus in carbohydrate reaches a state of equilibrium in approximately one hour, whereas the phosphorus in nucleoprotein may take up to 48 hours.

Malignant tumors contain more nuclei; therefore more nucleoprotein. It is the phosphorus in nucleoproteins that we wish to count and therefore more rewarding results will be obtained if the count is made at longer intervals after injection.

The instrument used to count the radioactivity must be placed directly over the lesion. Radioactive phosphorus is a pure

* From the Division of Ophthalmology, Department of Surgery, Stanford University School of Medicine. Supported by an institutional research grant from the American Cancer Society.

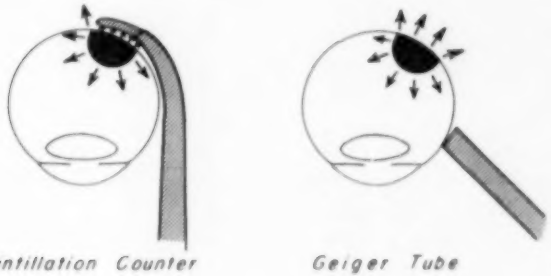

Fig. 1 (Bettman and Fellows). Comparison of scintillation and Geiger counters.

beta emitter. The beta rays have a maximum penetration of eight mm. and an average penetration of three mm. through tissue. If a Geiger-Müller tube is placed directly over the lesion, it will count every beta ray that gains access to it; but a Geiger-Müller tube of such a size and shape that it can be placed over a lesion situated in the posterior two thirds of the eyeball has not been available until the present time.

Figure 1 shows the Geiger-Müller tube designed for use over the eye, and Figure 2 shows the tube in position over the globe, contrasted with a scintillation probe which can be placed posteriorly. It will be readily seen that beta rays emanating from a lesion in the posterior two thirds of the globe will not be able to travel as far as the Geiger-Müller tube and consequently the efficiency of the tube for such a lesion is zero. The clinical incidence of these posterior lesions is relatively large—well over 50 percent of all intraocular lesions.

More recently a Geiger-Müller tube has been produced in a modified shape and size so that it can be inserted behind the eyeball. However, in producing these changes the efficiency of the instrument has been reduced to hardly 60 percent of the efficiency of the Geiger-Müller tube previously used.

Even for lesions situated more anteriorly, the counter must be placed directly over the lesion and not turned at an angle; otherwise beta particles emanating from the rectus muscles and varying thickness of uveal tissue will alter the count.[3]

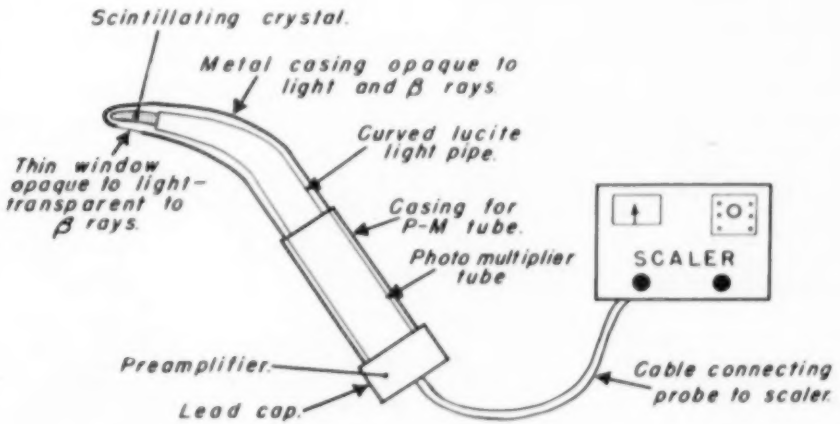

Fig. 2 (Bettman and Fellows). Diagram of scintillation counter.

It occurred to us that a scintillation counter might be developed for use directly over a tumor situated in the posterior portion of the globe. It is conceded that a Geiger-Müller tube may be a slightly more efficient instrument for counting beta particles when those particles can gain access to it, but, as pointed out above, the particles from a posterior lesion cannot gain access to it, and therefore in this instance the Geiger-Müller tube is totally inefficient. The smaller, curved Geiger-Müller tube for posterior use is not of very high efficiency.

A scintillation counter works on an entirely different principle than the Geiger-Müller tube. The latter consists of a metal cylinder filled with an inert gas and with a wire running down the center of the cylinder. The central wire acts as the anode and the metal wall acts as the cathode when a voltage potential is maintained across these electrodes. One end of the metal cylinder is covered only with a mica window through which a beta particle can enter. The beta particles produce ion pairs by collision. The pairs produce others and this process is repeated until an entire avalanche of ions is created and these register one count on the scaler.

The scintillation counter consists primarily of a material called a phosphor, capable of producing light when ionized by a radioactive particle. The light from the phosphor can be conducted around a curve, as from the posterior part of the eyeball, by a material such as polymethyl methacrylate (lucite perspex) which acts as a "light pipe."

Through the medium of the lucite light pipe the light is conducted to a photomultiplier tube. On entering the tube the light strikes the photo-cathode where the light energy causes an electron to be given off. Within the tube this electron strikes a dynode and causes several electrons to be given off. These strike other dynodes, and the entire process is repeated so that the original electron is multiplied many times. The current resulting from these many electrons is amplified and registers as one count on an automatic counting device known as a scaler (figs. 3 and 4).

Scintillation counters are widely used in physics and medicine, but none has been developed for use behind the eyeball. A series of experiments were conducted to develop a scintillation counter for use over the posterior portion of the globe. It was agreed that the following criteria must be fulfilled:

1. The instrument must be of such a size and shape that it can be inserted behind the globe through an opening in the conjunctiva.

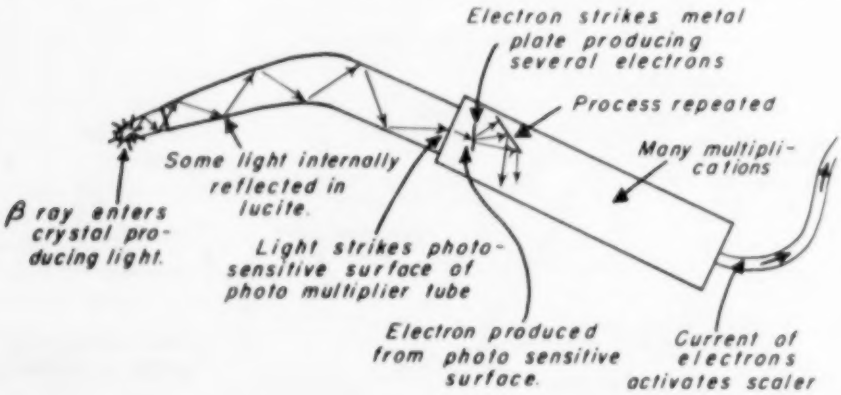

Fig. 3 (Bettman and Fellows). Diagram of how a scintillation counter works.

2. The phosphor must be one which does not readily deteriorate, is transparent to its own light emanations, and gives a strong enough pulse on stimulation with beta particles that the light can be conducted through curved lucite light pipe and present enough energy to stimulate a photomultiplier tube.

3. The entire instrument must be shielded from beta particles arising from extraocular tissues.

4. The instrument must be sterilizable or covered with a sterilizable material.

In order to fulfill these qualifications a number of experiments were performed to determine (1) the substance most suitable as a phosphor, (2) the proper size, shape, and thickness of the phosphor, (3) certain other effects such as the importance of a reflecting material around the phosphor, (4) the proper size and shape of the "light pipe" and light-tight container.

These subjects are summarized as follows:

1. *Determination of a suitable phosphor.* [Phosphors may be classified as (a) inorganic, (b) organic crystalline, (c) organic plastic and solution phosphors.[6]]

The most efficient inorganic phosphor is zinc sulfide. This substance could not be used because it is opaque to its own phosphoresence in any useful thickness. Sodium iodide is next in efficiency, but could not be used because it is deliquescent. All other alkali halides are less efficient. The next best is potassium iodide activated with thallium, but its efficiency was less than the organic phosphors and it presented the disadvantage of containing the natural isotope K_4O which is radioactive. The latter raised the background count to a disturbing degree.

Anthracene (Cl_4 H_{10}) is the most efficient of the organic crystalline phosphors, and, as will be seen from Table 1, was the most suitable of all phosphors that might be considered for this purpose. It only presented the disadvantage that individual pieces could not be worked readily from a large block. An organic plastic phosphor was found to be almost as efficient as the anthracene, and was used for much of the experimental work because pieces could be readily cut from a large block and shaped, polished, and worked as desired.

2. *Determination of size, shape, and thickness of the phosphors.** It is obvious that

TABLE 1

EFFICIENCY OF CERTAIN PHOSPHORS COMPARED WITH GEIGER-MULLER TUBE*

	Efficiency (per cent)
Geiger-Muller tube	100
Anthracene	85
Scintillating plastic	62
KI (thallium)	44

* These are only approximate and vary with the size of the phosphor.

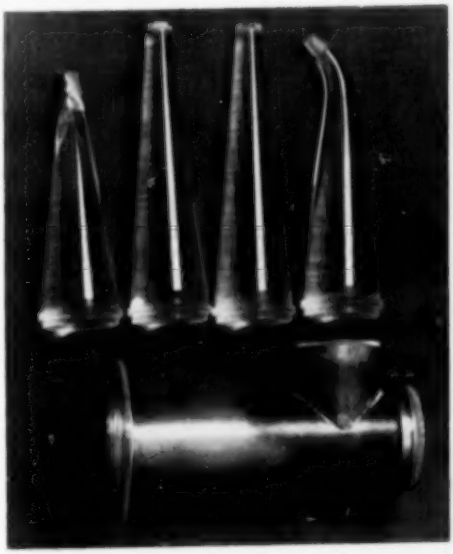

Fig. 4 (Bettman and Fellows). Photograph of experimental container with Lucite cones and various phosphors on their tip.

* In order to facilitate the testing of various phosphors an experimental container of one-mm. thick brass was made to attach to an RCA photomultiplier tube (#5819). At the end and side of the brass container of a Bakelite "window" was placed. The Bakelite "windows" were 25 mm. in

TABLE 2

EFFECT OF THICKNESS UPON EFFICIENCY

	Square (mm.)	Thick (mm.)	Efficiency (per cent)
Geiger-Muller tube			100.0
Scintillating plastic	7.5	6.0	82.8
Scintillating plastic	7.5	4.5	73.3
Scintillating plastic	7.5	3.0	69.8
Scintillating plastic	7.5	1.5	60.3

the larger the area of scintillating material exposed to a source of ionizing radiations, the higher will be the count. The effect of thickness had to be determined experimentally. Pieces of plastic material of the same area, but different thicknesses, were cemented to standard lucite light pipes and exposed to a standard sample of P^{32}. The results are shown in Table 2.

Table 2 demonstrates that a fourfold increase in thickness only increases the efficiency one fourth. It was obvious then that a thin phosphor which could readily be slipped behind the globe was almost as efficient as a thick, unwieldy phosphor.

In the foregoing experiments the phosphors were all tested while mounted with a large side cemented to the lucite. It was obvious that a more conveniently shaped instrument could be made if the phosphors were mounted on edge.

At first this seemed to result in too great a decrease in efficiency. The great decrease in efficiency was not due to the smaller surface of contact between the phosphor and the lucite light pipe. It was because the phosphor mounted on edge had to act as its own light pipe to conduct the light from the point of ionization to the lucite.

Experiments were undertaken to find out how an efficient instrument could be constructed.

From the experiments (table 3) we were assured that a conveniently shaped scintillator with a thin phosphor mounted on edge could be built with almost as much efficiency as a thick cumbersome phosphor mounted flat. In order to increase the efficiency of the conveniently shaped and mounted phosphor to this degree, it was necessary to make it wedge-shaped, with polished surfaces and wrapped in aluminum foil.

It was demonstrated that a convenient curve in the lucite light pipe did not decrease the efficiency as compared with a straight lucite light pipe.

On the basis of these experiments, a scintillation probe was constructed. It consisted of a phosphor of anthracene 7.5 mm. square and wedge-shaped, two mm. thick at the edge and three mm. thick at the surface of contact with the lucite light pipe. Each surface was polished. The lucite light pipe was made to fit the end of the phosphor wedge and of gradually increasing cross-sectional area with the other end ground to fit the photosensitive surface of a #5819

TABLE 3

EFFECT OF MOUNTING, POLISHING AND USE OF REFLECTOR ON EFFICIENCY

	Efficiency (per cent)
Geiger-Muller tube	100.0
Polished scintillating plastic, mounted flat	62.6
Wedge-shaped plastic (same size) mounted on edge, unpolished and without aluminum reflector	7.1
Same, polished but without aluminum reflector	28.7
Same, polished and with aluminum reflector over phosphor only	50.0
Same, polished and with aluminum reflector over phosphor and lucite light pipe	54.7

diameter, readily permeable to beta rays, but light-tight. The Bakelite windows were so positioned that various phosphors placed within the container were always at the same distance from a sample of P^{32} placed in contact with the Bakelite window. Several cone-shaped lucite light pipes of a standard size were made so that the phosphors could be quickly cemented to them. The light pipes were so shaped that their cross-sectional area constantly increased in size, and the ends were ground to the same curvature as the photosensitive surface of the photomultiplier tube. A picture of this experimental container and several lucite light pipes with phosphorus attached is shown in Figure. 5.

RCA photomultiplier tube. It was curved to make it convenient to place behind the globe.

A case of silver 0.6-mm. thick was made with a thin "window" of brass 0.012-mm. thick soldered to it. This thickness of silver prevents penetration of P^{32} beta particles, although the thin brass window permits almost unlimited entry of betas.

The case was constructed so as to be completely light-tight. The end of the case was machined to screw into the light-tight case surrounding the photomultiplier tube and preamplifier. Scintillations were counted on a scaling circuit.

A rubber sheath of the fingercot type was placed over the case enclosing the phosphor and lucite light pipe for sterility. A cloth sleeve was made to enclose the photomultiplier case in the same manner as it is used to cover an ophthalmoscope during a retinal reattachment operation. The use of the autoclaved rubber sheath and cloth sleeve rendered the entire instrument sterile, so that a surgeon might insert the radiosensitive tip through a conjunctival incision without fear of contamination.*

Fig. 5 (Bettman and Fellows). View of scintillation probe.

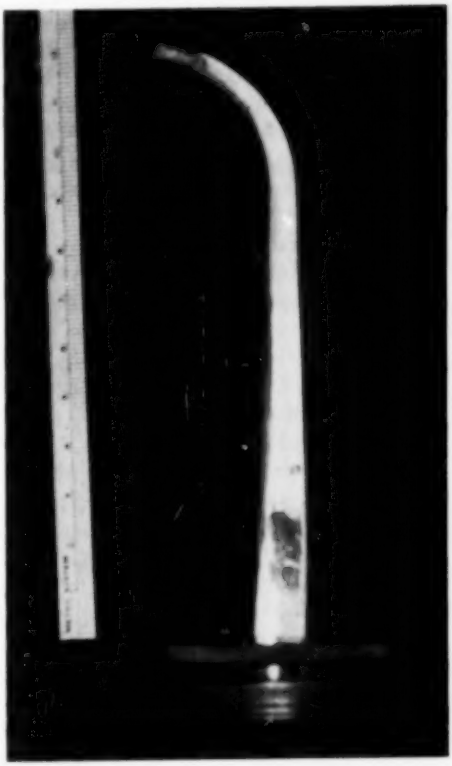

Fig. 6 (Bettman and Fellows). View of scintillation probe.

The anthracene scintillation probe finally constructed attained an efficiency of 93 to 100 percent of the straight Geiger-Müller tube. This scintillation counter has a much greater efficiency than the smaller curved Geiger-Müller tube made to use over the posterior globe. It is approximately of the curvature and thickness of the Arruga retractor used in retinal reattachment operations (figs. 6 and 7).

The scintillation counter presented in this article permits the ophthalmologist to obtain

* This scintillation counter consisting of the phosphor, light pipe, and light-tight case ready for connecting to the standard photomultiplier tube was made by the Parsons Optical Laboratory, 518 Powell Street, San Francisco.

a count of radioactivity from directly over a tumor situated in the posterior portion of the globe. This eliminates many of the inaccuracies encountered when a straight Geiger-Müller tube is used for this purpose as this tube is not of a size or shape that it can be placed anywhere near a posteriorly situated lesion.

The errors encountered with the straight Geiger-Müller tube are so gross as to render this counter totally useless for posterior lesions, as previously reported by us.[3] The small curved Geiger-Müller tube recently developed eliminates these errors, but it is less efficient than the scintillation probe. Under the conditions of a physics laboratory, the Geiger-Müller tube is a slightly more efficient instrument for the counting of beta particles than the scintillation counter. Under the conditions of a clinical diagnosis of a tumor situated in the posterior two thirds of the globe, the scintillation counter is definitely more efficient.

Summary

The use of radioactive phosphorus in the diagnosis of intraocular tumors may be a very useful clinical procedure provided (1) the counts are made long enough after the injection of P^{32} and (2) the counter is placed directly over the lesion.

The straight Geiger-Müller tubes available cannot be placed over a lesion in the posterior portion of the globe and are totally inefficient for this purpose. The newly developed curved Geiger-Müller tube is less efficient than the straight tube and less efficient than a scintillation probe.

A scintillation counter can be constructed of such a size and shape that it can be placed directly over such a posterior lesion.

Experiments established that an efficient instrument could be made of a phosphor of anthracene made in a wedge-shape with polished surfaces and cemented on edge to a lucite light pipe. The light pipe was curved and made of a gradually increasing cross sectional area and fitted to a photomultiplier tube. The phosphor and light pipe were wrapped in a reflector of aluminum foil and the whole was encased in a silver case impermeable to beta radiations except for a window of very thin brass to be placed directly over the lesion. Under conditions of a physics laboratory the scintillation counter had an efficiency of 93 percent of a Geiger-Müller counter, but under clinical conditions it was definitely more efficient.

2400 Clay Street (15).

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Discussion

Dr. Frederick C. Blodi: This new scintillation counter is certainly a valuable step forward in the diagnosis of intraocular tumors. However, a word of warning:

At the University of Iowa we have used scintillation counters—not curved ones and not ones that could be introduced behind the conjunctiva—in cases in which we could not find an appreciable

difference in the uptake of radioactive phosphorus in one eye or the other in a suspected intraocular tumor.

I have in mind especially one case of a young child who came to the clinic with a diagnosis of retinoblastoma. The uptake of radioactive phosphorus as counted with the Geiger counter was the same in one eye as in the other. When the child was tested with the scintillation counter we found an increase in the suspected eye, and we enucleated the eye. The histologic result was a retinal detachment without a tumor.

DR. J. W. BETTMAN (closing): I want to emphasize once more, if I may, that the scintillation counter is not superior to a Geiger tube for the counting of beta rays. The superiority of the scintillation counter lies in the fact that it can be curved and slipped behind the globe without loss of much efficiency.

An instance in which a straight scintillation counter was used, would really have no bearing on the particular type of lesion of which we are speaking here.

THE MECHANISM OF THE FALL IN INTRAOCULAR PRESSURE INDUCED BY THE CARBONIC ANHYDRASE INHIBITOR, DIAMOX*

BERNARD BECKER, M.D.
Saint Louis, Missouri

The recent introduction of such potent carbonic anhydrase inhibitors as Acetazolamide (Diamox)[1] has made possible the study of the effects of such inhibition in man. It soon became apparent that effective carbonic anhydrase inhibition prevented renal tubular reabsorption of sodium, bicarbonate and potassium, resulting in a marked diuresis, and inhibited pancreatic and gastric secretion. Most important from the ophthalmologic point of view was the reversible fall in intraocular pressure induced by systemic carbonic anhydrase inhibition.[2]

Since this finding has led to a rather extensive clinical trial of Diamox in the treatment of the glaucomas, it is of prime importance to determine the role of carbonic anhydrase in the maintenance of intraocular pressure and the site and mode of action of its inhibition.

Does Diamox act directly on the eye or is the induced lowering of intraocular pressure dependent upon the renal effect and its associated alterations in circulating electrolytes?

Does Diamox lower intraocular pressure by altering the outflow mechanism or does it inhibit secretory activity of the ciliary body?

It is the purpose of this report to summarize the results of recent clinical observations and animal experiments designed to answer these questions. Although definitive answers cannot be formulated yet, it would appear that Diamox lowers intraocular pressure by directly inhibiting the secretion of aqueous by the ciliary body. It is hoped that these tentative conclusions will lead to a more rational approach to the utilization of Diamox in the therapy of the glaucomas.

METHODS

Albino and pigmented rabbits (Haskins Rabbitry, St. Louis), weighing two to three kg. and maintained on Purina rabbit pellets, were used in these experiments. Mongrel dogs weighing 6.0 to 11 kg. were kept on a diet of Purina dog chow and canned horse meat.

All measurements of intraocular pressure in animals were made with a Schiötz to-

* From the Department of Ophthalmology, Washington University School of Medicine, and the Oscar Johnson Institute, Saint Louis, and the Wilmer Ophthalmological Institute of The Johns Hopkins Hospital, Baltimore. Supported in part by a grant to Washington University School of Medicine from the Alfred P. Sloan Foundation, Inc., upon recommendation of the Council for Research in Glaucoma and Allied Diseases. Neither the Foundation nor the Council assumes any responsibility for the published findings of this study.

nometer following local anesthesia with tetracaine (0.5 percent). No attempt was made to convert scale readings to pressure in mm. of mercury in dog or rabbit experiments. All animals were trained by repeated tonometry until repeated measurements could be duplicated to within one scale unit. A significant fall in intraocular pressure was arbitrarily set at three or more scale units. Most animals respond to Diamox with a change of four or five scale units. Animals which could not be successfully trained for tonometry or which did not give clear-cut significant responses to Diamox were discarded.

Intraocular pressure was measured in patients by Schiøtz or electronic tonometers. All tonography was carried out with a Mueller electronic tonometer connected to a Leeds & Northrup or a Brown recorder.

The sodium salt of Diamox* was used for all intravenous injections in concentration of 33 mg./cc. Diamox was administered orally in tablet form (50 and 250 mg. tablets). Ammonium chloride was given orally (500 mg. tablets) or subcutaneously (rabbits) in concentration of 7.5 mg./cc.

A. Site of action of diamox

1. *Can the fall in intraocular pressure occur in the absence of a renal effect?*

It was soon apparent that the repeated oral administration of Diamox every four to six hours to patients resulted in a cessation of the diuretic effect after the first 24 to 36 hours. Under these circumstances significant ocular hypotension persisted in spite of the absence of diuresis, suggesting an effect of Diamox on the eye that is independent of the renal action.

Maren[3] has demonstrated that preadministration of ammonium chloride to dogs prevents the diuretic action of Diamox. Attempts were, therefore, made to determine the effect of Diamox on intraocular pressure in animals and patients where the diuretic effect had been blocked by preadministration of ammonium chloride.

The administration of oral ammonium chloride on nine occasions to seven dogs in doses adequate to prevent diuresis (250 to 1,000 mg./kg.) failed to prevent a full fall in intraocular pressure to a challenge of oral Diamox (25 to 50 mg./kg.) (table 1). Similarly, ammonium chloride (75 to 200 mg./kg.) injected subcutaneously into 13 rabbits failed to alter significantly their ocular response to Diamox administered intravenously (100 mg./kg.) (table 2).

Eleven patients given 100 mg./kg./day of ammonium chloride by mouth demonstrated just as adequate a depression of intraocular pressure on chronic Diamox as they did before ammonium chloride administration. Furthermore, by the preadministration to three patients of ammonium chloride for 24 to 36 hours (2.0 gm. every four to six hours) the ocular effects could be obtained following Diamox administration without significant diuresis or rise in urinary pH. This provides additional evidence for the lack of dependence of the ocular effect on the diuresis. It should be noted that severe acidosis alone will lower intraocular pressure in patients (tables 1 and 2).

Most convincing evidence that the ocular hypotension induced by Diamox can occur independently of the renal effect was obtained by experiments on nephrectomized rabbits. Forty rabbits were nephrectomized 18 to 24 hours before the administration of Diamox. In every instance intraocular pressure fell following intravenous administration of Diamox. The fall was more pronounced than before nephrectomy and could be obtained with doses of Diamox as small as 4.0 to 8.0 mg./kg. Ocular hypotony persisted for the life of the animal. Thus, the ability of Diamox to lower intraocular pressure in rabbits without diuresis appears established.

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*The Diamox used in these experiments was supplied through the courtesy of Dr. James W. Gallagher of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

TABLE 1

EFFECT DIAMOX-AMMONIUM CHLORIDE ON INTRAOCULAR PRESSURE* IN DOGS

Dog No.		Normal	Diamox†	NH_4Cl(mg./Kg.)‡	NH_4Cl+Diamox§
1	RE	5.0	9.0	5.5 (1000)	10.0
	LE	5.0	9.0	5.5	10.0
3	RE	6.0	10.5	7.0 (250)	14.0
	LE	6.0	10.5	7.0	14.0
4	RE	7.5	11.0	8.0 (330)	11.0
	LE	7.5	11.0	8.0	11.0
4¶	RE	8.0	—	9.0 (1000)	13.0
	LE	8.0		9.0	13.0
5	RE	10.0	13.0	11.0 (250)	14.0
	LE	10.0	13.5	11.0	14.5
8	RE	7.0	10.0	6.5 (300)	10.5
	LE	7.0	10.5	6.5	10.5
9	RE	9.5	13.0	9.5 (250)	12.5
	LE	10.0	13.0	9.5	13.0
9¶	RE	9.0	—	9.0 (650)	12.5
	LE	9.0		9.5	12.5
10	RE	8.0	13.5	8.5 (250)	13.0
	LE	8.0	13.5	8.5	13.0

* All pressures are expressed as tonometer scale readings (Schiøtz).
† Diamox given in oral dose of 25–50 mg./kg. two hours before pressure measurements recorded in this column.
‡ NH_4Cl given orally in dose indicated 48 hours after preceding Diamox and after intraocular pressure returned to original values.
§ Oral Diamox (as in †) given one hour after oral NH_4Cl (of ‡).
¶ Dog tested twice at different dose levels of NH_4Cl.

TABLE 2

EFFECT DIAMOX-AMMONIUM CHLORIDE ON INTRAOCULAR PRESSURE* IN RABBITS

Rabbit No.		Normal	Diamox†	NH_4Cl (mg./Kg.)‡	NH_4Cl+Diamox§
4	RE	4.0	7.5	3.0 (75)	7.0
	LE	4.0	7.0	3.0	7.0
3	RE	3.75	—	3.0 (75)	6.25
	LE	3.0		3.0	6.0
5	RE	4.0	—	3.5 (75)	7.0
	LE	3.5		3.5	7.5
6	RE	3.0	7.0	3.5 (75)	7.0
	LE	3.0	7.5	3.0	7.0
37	RE	2.5	5.5	3.5 (75)	7.5
	LE	3.0	6.0	4.0	7.0
41	RE	2.0	7.5	1.5 (75)	6.5
	LE	2.0	6.5	1.5	5.0
50	RE	5.5	12.0	— (75)	12.0
	LE	5.0	12.0		12.5
45	RE	6.0	11.0	7.0 (200)	10.0
	LE	6.0	11.5	7.0	10.0
13	RE	9.0	—	10.0 (200)	15.0
	LE	9.0		10.0	14.0
48	RE	4.5	7.5	6.0 (200)	9.5
	LE	4.0	9.0	6.0	9.5
106	RE	5.5	—	6.5 (200)	12.5
	LE	6.0		7.0	13.0
205	RE	5.0	10.0	6.0 (200)	11.5
	LE	5.0	10.0	6.5	10.0
205	RE	5.0	9.0	6.5 (200)	10.0
	LE	5.0	8.5	6.0	11.0

* All pressures are expressed as tonometer scale readings (Schiøtz).
† Diamox given in intravenous dose of 100 mg./kg. (33 mg./cc.), 30 minutes before pressure measurements recorded in this column.
‡ NH_4Cl given subcutaneously (7.5 mg./cc.) 24–48 hours after intraocular pressure had returned to normal.
§ Intravenous Diamox (as in †) given one hour after subcutaneous NH_4Cl (of ‡).

2. Does Diamox act directly on the ciliary body?

Wistrand[4] has demonstrated that carbonic anhydrase is present in the anterior uvea of rabbits. Green and co-workers[5] have reported over 90-percent inhibition of this carbonic anhydrase activity following intravenous Diamox. This suggests the ciliary body as a possible site for the action of carbonic anhydrase inhibitors. The rapidity of the fall in intraocular pressure (five to 10 min.) following intravenous administration of Diamox to the rabbit or man also suggests a direct effect on the eye. However, attempts to lower intraocular pressure by the local or subconjunctival application to the eye of carbonic anhydrase inhibitors have met with little success.[5] This may be a matter of lack of penetration of the inhibitor to the secretory site.

It may be concluded, therefore, that the fall in intraocular pressure induced by Diamox is not dependent on renal action and that there is suggestive evidence that Diamox acts directly on the ciliary body.

B. Mechanism of action of Diamox

Not only is carbonic anhydrase present in the ciliary body, but there is also evidence that the enzyme plays a role in the secretion of the aqueous humor. Thus, Kinsey[6] found a 15-percent excess of bicarbonate in the anterior chamber as compared to the plasma and even a much greater excess (42 percent) in posterior chamber fluids of the rabbit eye. He postulated that bicarbonate might play a pilot role in the formation of the aqueous humor. Although the intimate role of carbonic anhydrase in this secretory process has not yet been elucidated, it is reasonable to assume that the inhibition of this enzyme would interfere with the production or availability of bicarbonate and thus inhibit the formation of aqueous. This working hypothesis finds confirmation from the manner in which Diamox lowers intraocular pressure in rabbits and patients.

In rabbit experiments, Linnér and Friedenwald[7] have noted that Diamox induces a delay in the appearance time of intravenously injected fluorescein. Thus, these observers

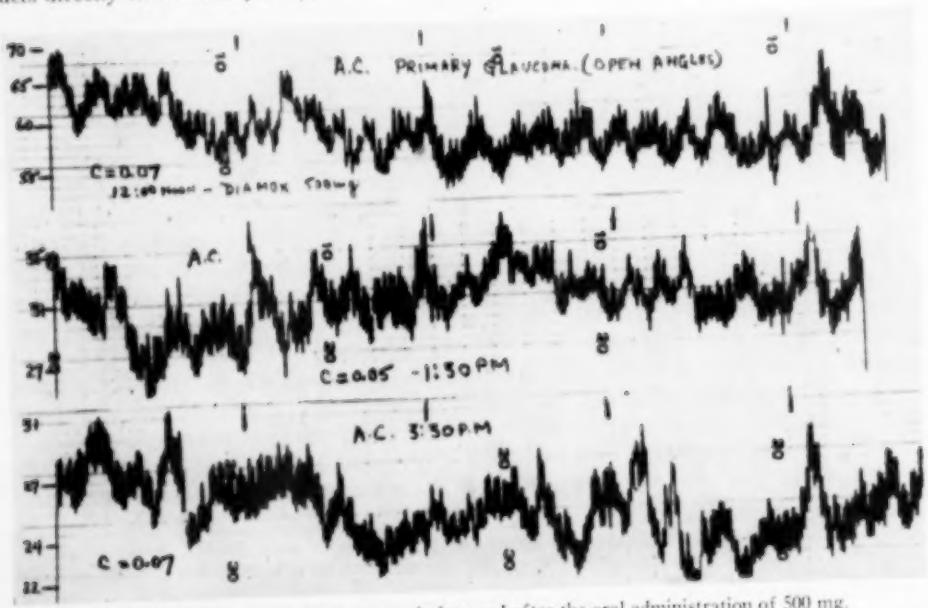

Fig. 1 (Becker). Repeated tonograms before and after the oral administration of 500 mg. Diamox to a patient with open-angle glaucoma.

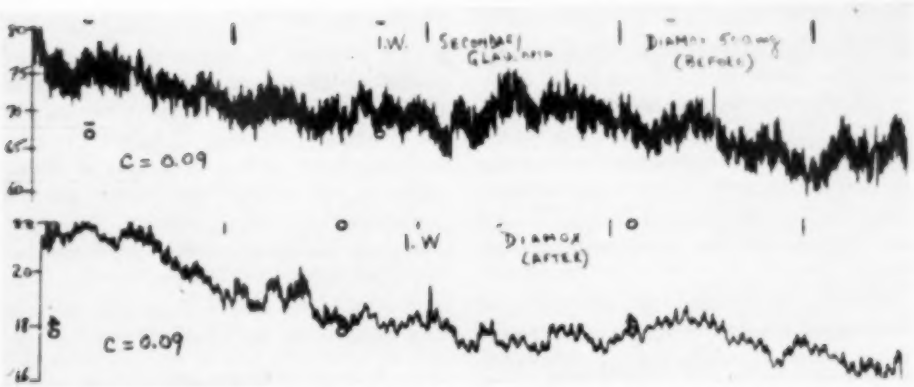

Fig. 2 (Becker). Tonograms before and after 500 mg. Diamox in a secondary glaucoma.

noted that the average fluorescein appearance time for 10 large rabbits was 133 seconds. If care is taken to eliminate those rabbits in which marked changes in iris permeability are present, nephrectomy alone does not affect fluorescein appearance time significantly. However, the administration of Diamox to the same 10 animals following nephrectomy resulted in a prolongation of the fluorescein appearance time to an average of 228 seconds.

Demorest and Becker[8] have confirmed this delay in fluorescein appearance time in rabbit eyes induced by Diamox administration. In this latter series the average fluorescein appearance time for 17 intact, pigmented, small (2.0 kg.) rabbits was 63 seconds. Eighteen hours after nine of these animals were nephrectomized, the average time of appearance was 64 seconds. However, when the remaining eight rabbits were similarly nephrectomized and received 100 mg./kg. of Diamox

TABLE 3

EFFECT OF ORAL ADMINISTRATION OF 500 MG. DIAMOX ON INTRAOCULAR PRESSURE AND FACILITY OF OUTFLOW
(16 eyes of 10 patients)

Patient	Diagnosis	P_1	P_2	C_1	C_2
M.N.	Normal	15	11	0.22	0.23
V.D.	Normal	17	12	0.22	0.25
	Normal	19	13	0.20	0.20
B.B.	Normal	18	13	0.30	0.32
	Normal	18	14	0.28	0.28
P.M.	Normal	17	13	0.26	0.26
	Normal	17	14	0.25	0.23
M.W.	Open angle glaucoma	55	25	0.07	0.05
	Open angle glaucoma	51	25	0.07	0.05
A.C.	Open angle glaucoma	65	27	0.07	0.07
C.H.	Open angle glaucoma	35	25	0.08	0.09
I.W.	Secondary glaucoma	76	24	0.09	0.09
	Secondary glaucoma	54	21	0.10	0.11
F.K.	Secondary glaucoma	33	22	0.07	0.06
	Normal	15	12	0.14	0.16
C.G.	Secondary glaucoma	55	27	0.08	0.10

P_1 = Intraocular pressure before Diamox.
P_2 = Intraocular pressure after Diamox.
C_1 = Facility of outflow before Diamox.
C_2 = Facility of outflow after Diamox.

intravenously, fluorescein was not visible in the anterior chamber until an average of 104 seconds.

Data have also been accumulated to demonstrate that Diamox will produce a similar delay in fluorescein appearance time in normal and glaucomatous eyes of some patients.[8] These findings suggest that Diamox induces a decrease in the rate of inflow of aqueous humor.

Clinical observations in patients reveal that Diamox administration is not accompanied by any change in pupillary size. Furthermore, the fall in intraocular pressure induced by Diamox appears to be independent of, and in addition to, the pressure-lowering effects of miotics and filtering procedures.

Most convincing evidence to date on the mechanism of action of Diamox is obtained by repeated tonography following short-term Diamox administration. As indicated in Figures 1 and 2, Diamox reduced the intraocular pressure in eyes with primary and secondary glaucoma, but the tonographic tracings remained flat. Table 3 summarizes the effects of Diamox on intraocular pressure and facility of outflow in 16 eyes of 10 patients. It will be noted that Diamox lowered intraocular pressure without significant change in facility of outflow. Such findings are consistent with the hypothesis that Diamox inhibits the inflow of aqueous into the eye, both normal and glaucomatous.

Summary

The experimental and clinical evidence suggest that the carbonic anhydrase inhibitor, Diamox, lowers intraocular pressure in man, the dog, and the rabbit by a direct effect on the eye, probably an inhibition of the formation of aqueous humor by the ciliary body.

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Discussion

Dr. Harry Green: Dr. Becker is to be commended for his continued interest in the experimental evaluation of the action of Diamox. It is true, as Dr. McDonald has pointed out, that the experimental observations Dr. Becker has presented make it difficult to disagree with the conclusions drawn therefrom.

However, at Wills Eye Hospital we have been conducting experimental tests with the use of Diamox on rabbits, and I would like to present some of our data which I believe lead to conclusions that suggest another possible explanation concerning the action of Diamox.

Our experimental observations were along two essential lines, namely, the effect of Diamox upon the carbonic anhydrase activity of the ciliary body and iris, and also the effect of Diamox on the bicarbonate levels of both the anterior aqueous humor and the blood plasma.

Two routes of administration were used—one intravenous and the other subconjunctival. The subconjunctival injection of 10 mg. of Diamox led to a complete inhibition of the carbonic anhydrase of the ciliary body and iris within 15 minutes, lasting for at least 30 minutes.

Intravenous injection of about 30 mg. of Diamox

per kg. of body weight of rabbit resulted in a time inhibition curve which was roughly an inverted V, with the peak of inhibition reached at about 30 minutes and representing 93-percent inhibition of the total carbonic anhydrase activity of the tissue.

In both cases we did not observe a fall in intraocular pressure. However, we were able to detect the presence of Diamox in the fluid of the anterior chamber.

After subconjunctival injection of 10 mg. of Diamox, no drop in the bicarbonate level occurred in either the anterior aqueous humor or the blood plasma for approximately 60 minutes. With larger doses, however, and perhaps over a longer period of time, there was a fall in the bicarbonate level of the anterior aqueous, but there was a corresponding fall in the bicarbonate level of the blood plasma, at the same time.

The results with the intravenous administration of Diamox and its effect on the bicarbonate level in both tissue fluids is shown on the slide.

(Slide) The upper curve is the bicarbonate level of the aqueous humor in the anterior chamber, and the lower curve is the bicarbonate level in the blood obtained from the same rabbit practically simultaneously with the tap of the anterior chamber. Time periods are as indicated.

We believe it is very significant that the two curves are practically parallel, that is, the fall and the rise in the bicarbonate level are practically the same for both fluids.

These results would tend to suggest that the bicarbonate level in the anterior aqueous is largely determined by the bicarbonate level fluctuations in the blood plasma, and it would tend to indicate that the mechanism for the maintenance of the difference between the bicarbonate levels in the two fluids is independent of the action of Diamox and is not determined by the carbonic anhydrase activity of the ciliary body and iris.

In conclusion, I would like to add that our observations are difficult to reconcile with the presently accepted view that the bicarbonate level in the posterior chamber is elaborated by an active transfer mechanism, presumably by the ciliary body.

DR. P. ROBB MCDONALD: I am sure that most of you are aware of the several clinical conditions for which Diamox may be used. Probably you do not have the complication of a shallow anterior chamber following cataract surgery! This is not an original observation of mine, but Diamox will frequently relieve this condition, especially those cases in which the chamber becomes shallow on the second or third day. These are usually associated with a choroidal detachment.

DR. FREDERICK C. BLODI: I would like to elaborate on some of the statements about the local use of Diamox as presented by Dr. Becker and Dr. Green.

In Iowa City we were rather intrigued by these possibilities for some time, and we have the permission of Dr. Foss to report on his experiments.

We tried to use Diamox locally by topical administration in various concentrations and also by subconjunctival injection in various concentrations and frequencies, in a way similar to that mentioned by Dr. Becker and Dr. Green, without seeing any effect on the intraocular pressure. We then tried to inject Diamox intracamerally in various concentrations and with various frequencies. Again the result was absolutely negative as far as intraocular pressure was concerned.

However, it was interesting to see that if the concentration of Diamox was increased there was quite a damaging effect to the cornea. The cornea becomes edematous, not unlike the picture we saw the other day after the injection of streptokinase. The effect on the cornea is temporary, and it depends very much on the concentration of the drugs used.

DR. JOHN HARRIS: To the long list of failures of local use, I could also add the administration by iontophoresis, at least in animals. I have hesitated to use such a route in patients because one might accidentally induce a high local accumulation. So far, we have been unable to work out a satisfactory method for its chemical determination.

We have been concerned about the possibility of a high accumulation of Diamox within the eye because of some observations we have also made on the effect of the carbonic anhydrase inhibitors on the cation transport of the lens. They seem to inhibit cation transport quite effectively. Our experience has indicated that the ability to inhibit cation transport provides a rapid and reasonably accurate measure of the cataractogenic potential of an agent.

We also have done experiments very similar to those Dr. Green mentioned, and have observed a marked drop in the bicarbonate level of the arterial plasma and a concomitant drop in the bicarbonate content of the aqueous. In each instance, however, the decrease in aqueous concentration paralleled and seemed dependent on the plasma concentration as Dr. Green described.

We were unable to demonstrate any preferential drop in the bicarbonate concentration of the aqueous humor. By various local routes—by iontophoresis, subconjunctival injections, and so on—no change in the bicarbonate concentration was observed.

However, I do not feel that this mitigates against the conclusion that Dr. Becker has enunciated here. In many situations, both clinical and in the experimental animal, the drug is very similar to Dibenamine. At times Dibenamine may cause a drop in the bicarbonate concentration of the aqueous humor, but always we have found a similar and parallel drop in the bicarbonate concentration of the plasma.

However, if one measures the turnover of aqueous, particularly using glucose as the measuring tool, Dibenamine appears to reduce aqueous formation. Thus, the aqueous-forming mechanism attempts to maintain a relatively constant chemical composition with respect to plasma, and the induced pressure drop which Dr. Becker has indicated is not so much due to a change in composition as it is a change in total elaborated volume of an

aqueous of relatively constant composition with respect to plasma. This concept fits our data with Dibenamine and our data with Diamox.

Dr. Henry F. Allen: We believe that primary glaucoma is due to an obstruction to outflow while the input remains constant. We believe that Diamox, without affecting the outflow, reduces the inflow of aqueous. I would like to ask if there is a quantitative difference in the response to Diamox of normal eyes—eyes with normal tension—and eyes with high tension, and, if there is a quantitative difference, whether it can be reconciled with those two theories.

Dr. Harry Green: I would like to make just one observation relative to the observation of Dr. Blodi namely, that the intracameral injection of Diamox in high concentrations leads to a local edema of the cornea.

I wonder if that may be due to the relatively high alkaline pH of a water solution of sodium Diamox which exceeds that of 9.0. In our experiments we have neutralized the sodium Diamox to a pH of 8.8 to 9.0, and have not noticed any local inflammatory effects in the eye.

Dr. Bernard Becker (closing): First, let me thank Dr. Green, Dr. Blodi, Dr. Harris, and Dr. Allen for their interesting discussions. That was one of the purposes of presenting this material—to elicit such discussion.

In answer to Dr. Green, I am very glad to hear that he has confirmed many of our findings. The evidence he presents that systemic Diamox administration does inhibit the carbonic anhydrase of the ciliary body provides another postulated link in the chain of arguments used as to the mechanism of action of Diamox.

Dr. Harris asked about a method for Diamox. I would suggest that he speak to Dr. Green who informed me that he is about to publish a method for determining Diamox.

The failure to find a fall in intraocular pressure after local administration of Diamox corresponds to the lack of fall of bicarbonate ion in the anterior chamber under these circumstances. This remains one of the difficulties that we cannot explain. I would still like to look at this in terms of a failure of Diamox to penetrate to the secretory site. As Dr. Friedenwald pointed out at the Wilmer meeting, Diamox may act on the stroma of the ciliary body, and it would be difficult to know whether it actually penetrates to that site. In fact, why or how drugs penetrate to the stroma of the ciliary body is poorly understood.

There is one point that is well worth making because it has led to a lot of work that has been all for naught. There are rabbits responsive and rabbits unresponsive to Diamox. This may sound like a minor point, but actually it turns out to be very important. Dr. Kinsey has spent a great deal of time and effort on the problem of unresponsive rabbits. Ballintine in Cleveland has also encountered unresponsive rabbits. For some reason that has not yet been explained, although all dogs respond and almost all people respond, there are certain groups of rabbits which, either because of their age, their diet, their heredity, or some other condition, do not respond with a fall in intraocular pressure to Diamox. I think it is rather unfortunate that Dr. Green is working with a group of rabbits that do not respond to Diamox with a fall in intraocular pressure. It may account for his failure to alter aqueous bicarbonate levels more dramatically. We have been more fortunate both in Baltimore and in St. Louis in having responsive animals.

The point has been made by Dr. Green and by Dr. Harris that the fall in plasma bicarbonate and the fall in anterior chamber bicarbonate always parallel one another. We are dealing with a multiplicity of effects in systemic administration of Diamox. It alters the amount of carbon dioxide blown off in the lung. There is a large effect on the kidney tubule, increasing the amount of bicarbonate excreted. The renal effect can be eliminated by nephrectomy. In such nephrectomized responsive rabbits, Diamox induces a fall in aqueous bicarbonate with little change in plasma bicarbonate. This is suggestive evidence that Diamox alters the production or entrance of bicarbonate into the eye.

We also have noted corneal damage upon injecting Diamox into rabbit eyes, but only if alkaline solutions are used.

I think it is very important, as Dr. Harris has indicated, for more work to be done on the effects of Diamox on the lens. There is an enormous concentration of carbonic anhydrase in the lens, and, as far as I am aware, no one has established the functional role of the enzyme in the lens.

The only other point I want to make is in answer to Dr. Allen's question. One can quantitate the effects of Diamox from the tonographic tracings. As you know, tonography can be used as a method for determining the rate of flow of aqueous, as Grant has pointed out. Although there are many sources of error inherent in the method, when it is used repeatedly on the same eye, many of the errors cancel out and one can determine how much one has inhibited flow of aqueous by the administration of Diamox.

When one does so in the series of patients such as was presented here, one finds that there is essentially the same amount of inhibition of flow in normal eyes as there is essentially the same amount of inhibition of flow in normal eyes as there is in eyes with glaucoma. The amount of inhibition of flow that is induced by the administration of 500 mg. of Diamox varies from approximately 50 to 70 percent. However, 50 percent inhibition of flow in a normal eye may lower the pressure from 18 to 14 mm. Hg, whereas, 50 percent inhibition of flow in a glaucomatous eye may lower the pressure from 60 to 35 mm. Hg.

STUDY OF PATENCY OF OPENINGS IN CORNEAS ANTERIOR TO INTERLAMELLAR PLASTIC ARTIFICIAL DISCS*

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This paper presents a continuation of work reported in 1952.[1] At that time it was shown that plastic discs of various diameters could be maintained in rabbit corneas in interlamellar fashion for indefinite periods. It was also shown that an opening in the corneal layer anterior to the plastic disc remained patent in one animal for one year until the natural death of the animal. Since then, plastic discs have been maintained in some rabbit corneas indefinitely (table 1) (fig. 1). Until recently, however, it has been impossible to maintain an opening in the anterior layer of a cornea for more than a short period. This report concerns the experiments conducted in an attempt to maintain patency of these openings.

BASIS FOR EXPERIMENTAL METHOD

As reported in 1952,[1] the anterior corneal opening which remained patent for one year had been formed inadvertently. It was thought then that the experiment could be repeated easily by means of trephining the anterior corneal layer, and that it would be necessary merely to find the optimal size hole and the optimal time of placement. This was not so. Nonetheless, it was decided to continue to center the experimental design around variation of operative procedure rather than to introduce other factors, such as growth inhibiting substances, for it had been without the agency of other factors that the first experiment had proved successful.

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VARIATION OF SIZE OF TREPHINE HOLE

Trephine holes of varying diameters were made in the anterior corneal layers after the placement of the discs. In each case, in approximately six to 27 days after the hole was trephined, a salmon-colored, slightly raised ring about two mm. in width would appear at the edge of the hole (fig. 2B). This ring would gradually advance toward the center of the hole (figs. 2A to F, inclusive) until after approximately 37 days the entire opening would be closed by newly formed tissue.

Grossly this tissue showed translucent and opaque areas, suggesting possibly a more symmetrical arrangement of new fibers in the translucent areas. It is seen in Table 2 that the size of the opening influences the time of closure very little; and in no case did a trephine hole, no matter how large, remain patent beyond 52 days (table 3).

TIME INTERVAL BETWEEN PLACING IMPLANT AND TREPHINING HOLE

It had been thought that the time interval between the placing of the plastic disc and the trephining of the hole might be a factor in maintaining patency. Approximately six to 26 days after the placement of the disc,

TABLE 1

RABBITS MAINTAINING INTERLAMELLAR PLASTIC DISCS WITHOUT EXTRUSION FOR LONGEST TIME INTERVALS, AS OF JUNE 16, 1954

Rabbit	Operative Date	Date of Death	Time in Place
7	1/31/52	11/20/53	1 yr., 9½ mo.
15	2/13/52	—	2 yr., 4 mo.
80A	5/25/50	9/ 9/53	3 yr., 3 mo.
234	10/29/52	—	1 yr., 7½ mo.

TABLE 2

RELATION BETWEEN SIZE OF TREPHINE HOLE IN CORNEAL LAYER ANTERIOR TO PLASTIC DISC AND TIME OF CLOSURE IN RABBIT EYES

Rabbit Number	Experiment Number	Size of Trephine (mm.)	Time of Closure After Operation (Days)
13	6	4	40*
25	1	4	50*
30	2	4	30
64	1	4	38
15	5	5	40
42	1	5	35
225	2	5	20
234	1	5	40
234	5	5	40*
234	6	5	30
49	1	5	33
51	3	5	27
51	4	5	41
55	1	5	20
61	1	5	48
74	1	5	50
75	1	5	38
77	1	5	35
77	2	5	43
78	1	5	50*
79	1	5	31
84	1	5	52
85	1	5	52
15	1	6	41
20	2	6	30
21	2	6	28
29	2	6	40
30	1	6	35
225	1	6	25
225	3	6	40
227	1	6	41
51	2	6	37
51	6	6	29
52	1	6	45
52	2	6	49
54A	1	6	36
74	2	6	50*
15	2	7	48
15	3	7	50
25	2	7	42
25	3	7	40
29	3	7	35
51	1	7	45
234	3	8	27

* Approximated.

large sheaths of vessels grow into the cornea from the limbus and are finally supplanted by several fine trunks. It was reasoned that by making the trephine hole before this ingrowth, the nutrition of the cells at the trephine edge might be impaired and thus inhibit or redirect their growth backward to the stroma and the peripheral holes.

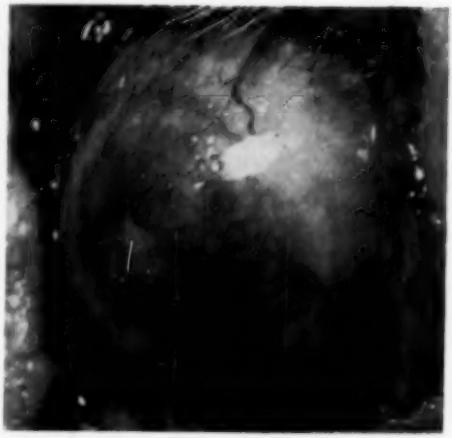


Fig. 1 (Stone). Photograph shows an interlamellar plastic disc which has been in place two years, four months as of June 16, 1954. Rabbit 15. Operative date, February 12, 1952. Photographed June 8, 1954.

As can be seen in Table 4, this hypothesis was not substantiated. The holes closed over in approximately the same period, 20 to 52 days, irrespective of the time they were made after the discs were inserted (table 5).

It was learned, nevertheless, that when trephining was done at the same time as, or within one week after the placement of the discs, they were more likely to extrude. This could be caused by three factors: (1) The stroma had not as yet invaded the peripheral holes; (2) barriers to infection had not as yet been erected (table 4, Rabbits 20, 46, 47,

TABLE 3

TIME OF CLOSURE IN RELATION TO SIZE OF TREPHINE HOLE IN RABBIT CORNEAS

Number of Rabbits	Size of Trephine (mm.)	Closure Time After Original Operation		
		Maximum	Minimum	Average
		(days)	(days)	(days)
4	4	50	30	40
19	5	52	20	38
14	6	50	29	38
6	7	50	35	43
1	8	27	27	27
		Over-all average		37

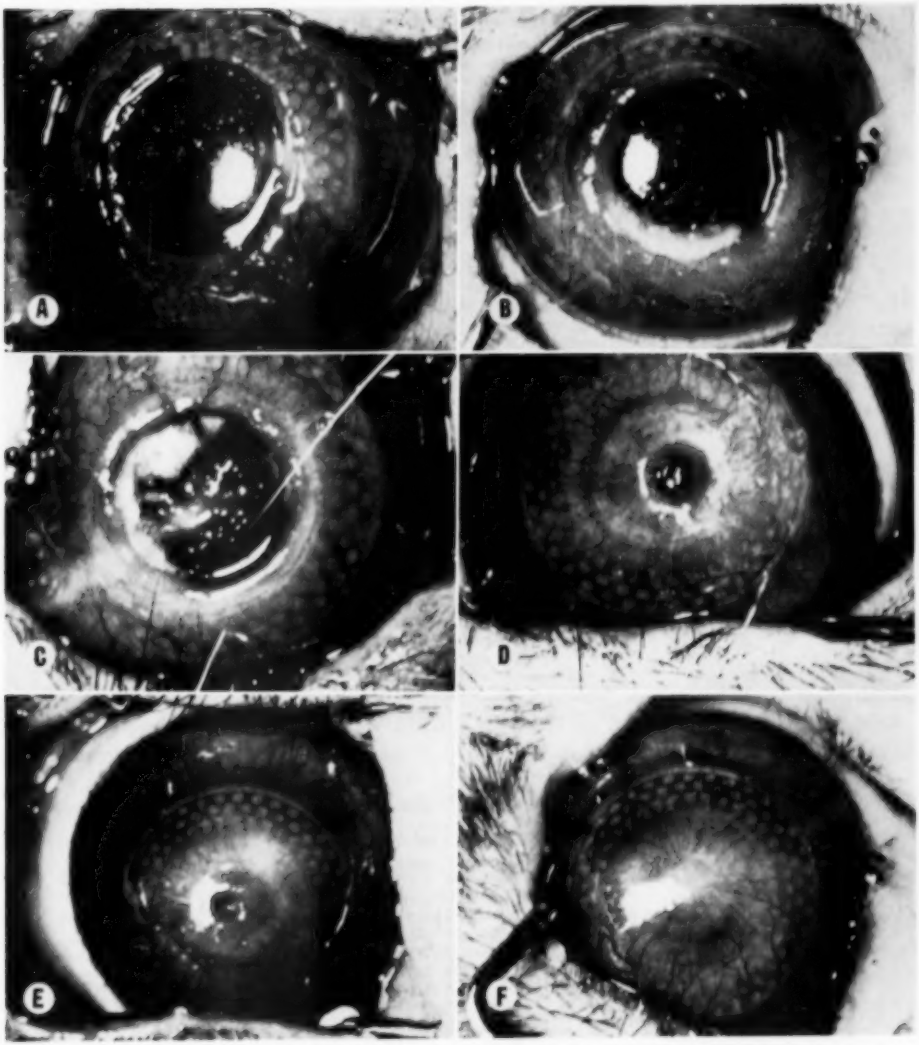


Fig. 2 (Stone). Photographs show closing of trephine hole in anterior corneal layer in front of plastic disc. Rabbit 21, Experiment 2.

(A) Two days postoperative, slight engorgement of vessels. (B) Eight days postoperative, distinct salmon-colored ring can be seen at edge of hole. (C) Thirteen days postoperative, salmon-colored edge has increased in thickness and width. (D) Twenty-one days postoperative, salmon-colored edge has nearly obliterated hole. (E) Twenty-three days postoperative. (F) Twenty-nine days postoperative, completely closed.

TABLE 4

RELATION OF TIME INTERVAL BETWEEN PLACING OF TREPHINE HOLE AFTER INSERTION OF PLASTIC DISC AND CLOSURE OF HOLE IN RABBIT CORNEAS

Rabbit Number	Experiment Number	Time after Original Operation Trephine Placed	Time of Closure after Trephine Placed (days)	Time of Extrusion after Trephine Placed (days)
45	1	Trephined during placement of disc		40
46	1	Trephined during placement of disc		20
47	1	Trephined during placement of disc		21
48	1	Trephined during placement of disc		30
49	1	Trephined during placement of disc	33	
50	1	Trephined during placement of disc		84
51	1	Trephined during placement of disc	45	
52	1	Trephined during placement of disc	45	88
52	2	Trephined during placement of disc	49	
53	1	Trephined during placement of disc		32
54A	1	Trephined during placement of disc	36	
58	1	Trephined during placement of disc		130
61	1	Trephined during placement of disc	48	
64	1	Trephined during placement of disc	38	
65	1	Trephined during placement of disc		50
66	1	Trephined during placement of disc		51
68	1	Trephined during placement of disc		25
69	1	Trephined during placement of disc		24
74	1	Trephined during placement of disc	50	
75	1	Trephined during placement of disc	38	
77	1	Trephined during placement of disc	35	
78	1	Trephined during placement of disc	50	
79	1	Trephined during placement of disc	31	
80B	1	Trephined during placement of disc		14
84	1	Trephined during placement of disc	52	
85	1	Trephined during placement of disc	52	
10	1	Trephined during placement of disc		23
11	1	Trephined during placement of disc		11
227	1	Trephined during placement of disc	41	
7	1	7 days		73
225	1	22 days	25	
25	1	25 days	50	
234	1	27 days	40	
55	2	1 mo., 7 days	20	
42	1	1 mo., 11 days	35	
61	2	1 mo., 29 days	51	
77	2	2 mo., 3 days	43	
55	3	2 mo., 4 days		7
74	2	2 mo., 20 days	42	
20	2	3 mo., 11 days	30	199
30	1	3 mo., 19 days	35	
225	2	3 mo., 22 days	20	
51	2	3 mo., 25 days	37	
234	3	4 mo., 5 days	27	
30	2	4 mo., 25 days	30	
51	3	5 mo., 2 days	27	
51	4	6 mo., 5 days	41	
29	2	6 mo., 12 days	40	
21	2	7 mo.	28	
25	2	7 mo., 17 days	42	
51	5	7 mo., 29 days	26	
15	1	8 mo., 11 days	41	
29	3	8 mo., 12 days	35	
51	6	8 mo., 25 days	29	
15	2	9 mo., 23 days	48	
225	3	9 mo., 28 days	40	
25	3	10 mo., 19 days	40	
234	5	11 mo., 23 days	40	
15	3	12 mo., 27 days	50	
234	6	14 mo., 26 days	30	
15	5	19 mo., 12 days	40	
15	6	22 mo., 21 days	40	

TABLE 5

RELATION OF TIME INTERVAL BETWEEN PLACING OF TREPHINE HOLE AFTER INSERTION OF PLASTIC DISC AND CLOSURE OF HOLE IN RABBIT CORNEAS

Number of Rabbits	Time after Original Operation Trephine Placed	Time of Closure after Trephine Placed (days)		
		Maximum	Minimum	Average
29	Trephined during placement of implant	52	31	43
4	7 to 30 days	50	25	36
12	1 mo. to 6 mo.	43	20	33
13	6 mo. to 1 yr.	48	28	37
4	1 yr. to 2 yr.	50	30	40

52, 58); (3) the stability of the implant had not been established and consequently there was slippage of the disc.

VARIATION IN OPERATIVE TECHNIQUE

The results of various operative techniques employed in producing the trephine hole are shown in Table 6. The technique in which the edge of the hole was made perpendicular to the corneal surface is not listed in the table. This procedure was used in the majority of experiments shown in Tables 2 and 4. The holes remained open for only short periods.

The possibility that epithelial cells might grow around the edge of the underlying stroma and back to the region of the peripheral holes, if given assistance, was explored. In Table 6, items 2, 3, and 4, the technique was such that the edge of the opening was angled with its posterior part directed peripherally by means of a scissor incision. In items, 5, 6, 7, and 8, Table 6, are listed the experiments in which the epithelium was undermined back to the area of the first row of holes by means of incising immediately beneath the epithelium, lifting the epithelial layer, and cutting the stroma at the desired peripheral distance with an angle knife.

The procedure in items 9 through 18, Table 6, was to trephine an opening wider than the first row of peripheral holes. It was thought that the advancing epithelial cells might grow to the stroma tissue, invading the peripheral holes, and produce a seal. In an attempt to augment this and possibly permit the ingrowth of epithelial cells before the invasion of holes by stroma, the tissue was evacuated from the first row of peripheral holes (table 6, items 19 through 22). In all of the foregoing experiments there was no significant influence on the time of closure of the anterior corneal opening.

In items 23 through 26, Table 6, cruciate incisions were made in the center of the anterior corneal layer. The flaps so formed were drawn posteriorly and peripherally, folded upon themselves, and secured with catgut in the region of the peripheral holes. This was an attempt to bring the epithelial cells at the edge of the trephine hole into closer contact with the peripheral holes. There was an increase in time of closure of two out of four of these experiments to nearly the upper limit of the previously established closure times, that is, 52 days. This was not thought to be significant.

In the next four experiments (items 27 through 30, Table 6), in placing the implant, the incision into the cornea was very superficial throughout in an attempt to include only the epithelial cells and their basement membranes. This might allow the epithelial cells to grow backward before the advancement of stromal tissue. There was a significant increase in the time of closure of the holes in this experiment in two of the four procedures. While the holes were still patent in this experiment, it was found that in the experiments listed in items 28 and 30, Table 6, the periphery of the disc eroded through from pressure necrosis between the disc and the overlying lids.

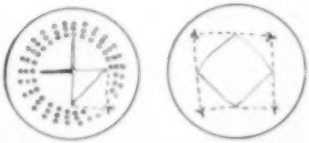
This observation initiated the procedure followed in experiments shown in items 31

TABLE 6

DIAGRAM OF VARIOUS OPERATIVE PROCEDURES USED IN CUTTING A HOLE IN CORNEAL LAYER ANTERIOR TO PLASTIC DISC AND RESULTS

Item Number	Operative Procedure	Rabbit Number	Exp. Number	Time of Closure after op.	Extrusion after Placement of implant.
1.	Straight Cut 	Experiments with this operative procedure not included. Majority of experiments in Charts 1 and 2 employed this method and are noted there.			
				DAYS	DAYS POST-OP
2	Angle Cut 	21	1	28	—
3		25	2	42	—
4		30	1	35	—
5	Undermined with Angle Knife 	29	1	40	
6		29	2	40	
7		30	2	30	—
8		42	1	35	
9	Trephined past first row of holes. 	15	2	48	—
10		227	1	41	
11		45	1	38	130
12		46	1	—	20
13		48	1	—	30
14		49	1	33	—
15		50	1	—	90
16		51	1	45	—
17		52	1	45	114
18		54A	1	36	

TABLE 6 *(Continued)*

Item Number	Operative Procedure	Rabbit Number	Exp. Number	Time of Closure after op.	Extrusion after Placement of implant.
				DAYS	DAYS POST-OP
19	Trephined past and with evacuation – first row holes. 	20	2	30	199
20		25	3	40	—
21		225	2	20	—
22		234	3	22	—
23	Cruciate incision with flaps sutured under. 	15	6	40	—
24		51	5	26	—
25		61	2	51	—
26		66	1	—	51
27	Very Superficial Incision 	234	1	40	
28		58	1	—	130
29		808	1	—	14
30		50	1	—	90
31	Thin-thick Incision 	61	1	47	—
32		62	1	OPEN 8 MONPO	—
33		65	1	—	72

through 33, Table 6. The center of the anterior corneal layer was dissected very superficially for two to three mm. from the edge of the trephine hole after it was cut. A deep corneal dissection was made from the periphery inward. The two planes were joined. The overlying tissue was then thick peripherally and thin centrally. The disc was slipped under these layers. The trephine hole was made at the time of the original operation.

In one of these latter experiments, item 32, Table 6, Rabbit 62 (fig. 3), the trephine hole was still patent as of June 16, 1954 (submission date of this paper), eight months after the original operation and trephining. One additional change was made in the procedure in this experiment, that is, the plastic implant itself was increased in central thickness.

Thickness of plastic implant

Prior to the experiment performed in Rabbit 62, the shape of the plastic implant had been altered continuously, but the thickness

TABLE 7

RABBIT CORNEAS FITTED WITH 1.5-MM. THICK IMPLANTS IN INTERLAMELLAR FASHION. TREPHINE HOLES (4.0 MM. TO 5.0 MM. IN DIAMETER) PLACED IN CORNEAL LAYER ANTERIOR TO PLASTIC DISC AT TIME OF INSERTION OF DISC

Rabbit	Operative Date	Result	Extrusions and Reasons (days postoperative)
60	9/23/53	—	40—eye infected
62	10/14/53	Open as of 6/16/54, 8 mo.	—
65	10/19/53	—	50—erosion
66	10/26/53	—	51—erosion
73	2/ 8/54	Open as of 6/16/54, 4 mo., 8 days	—
81	3/ 2/54	Open as of 6/16/54, 3 mo., 14 days	—
82	3/ 4/54	Closed, 2 mo., 23 days	—
83	3/ 4/54	Open as of 6/16/54, 3 mo., 12 days	—
87	3/15/54	Open as of 6/16/54, 3 mo., 1 day	—
88	3/17/54	—	9—eye infected

had remained relatively constant, that is, 0.25 mm. The thickness of the disc in Rabbit 62 was increased to 1.5 mm. in the center and the disc was reshaped.

When it was found that the anterior trephine hole in Rabbit 62 was destined to remain patent for longer than the usual period, rabbits noted in Table 7 were fitted with 1.5-mm. discs. The operative procedure in these entailed placing of the disc at moderate depth in the stroma and, at the same time, making a trephine hole in the center of the anterior layer.

As can be seen in Table 7, the opening in the anterior cornea of Rabbit 73 (fig. 4) has remained patent four months, eight days, as of June 16, 1954, the opening in the anterior layer of the cornea of Rabbit 81 (fig. 5) has remained patent three months, 14 days as of the same date: in Rabbit 83 (fig. 6) the opening has remained patent three months, 12 days as of June 16, 1954; and in Rabbit 87 (fig. 7) the opening has remained patent three months, one day as of June 16, 1954. With the same technique, extrusion occurred as a result of infection or erosion in Rabbits 60, 65, 66, and 88 (table 7). Rabbit 82 is of interest for, with presumably the same plastic

Fig. 3 (Stone). Rabbit 62. Trephine hole in corneal layer anterior to plastic disc has remained patent eight months as of June 16, 1954. Operative date, October 14, 1953. Photographed June 8, 1954. Disc of 1.5-mm. thickness at center.

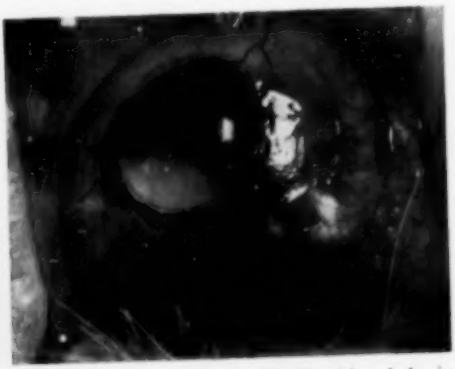


Fig. 4 (Stone). Rabbit 73. Trephine hole in corneal layer anterior to plastic disc has remained patent four months, eight days, as of June 16, 1954. Operative date, February 8, 1954. Photographed June 8, 1954. Disc of 1.5-mm. thickness at center.

disc and the same technique, the anterior hole closed in two months, 23 days (fig. 8, A, B, and C).

To evaluate the effect of reshaping the implant, discs with approximately the same shape as those placed in the experiments in Table 7 but with a thickness of 0.25 mm. throughout were placed in Rabbits 64, 89, and 90. Thus far in Rabbit 64 (fig. 9) the hole has grown over in 38 days. This has not occurred in the other rabbits but it is too early to draw any conclusions about the ultimate outcome.

Preparation and Handling of Plastic Discs

Minute care and close supervision are necessary in the preparation of these discs. Small details in their handling often mitigate against the eventual result. Such insignificant factors as incomplete filtering of wash water may precipitate microscopic rust specks on the disc and produce foreign-body reactions. Minute oil films may come from inadequately filtered compressed air lines. Lack of rigid control of sterilizing temperatures permits slight variations in the shape of the implants sufficient to produce pressure necrosis in the cornea. Polishing materials and procedures are not utilized because of the possibility of imbedding microscopic foreign bodies in the plastic.

Differences in the raw stock from the manufacturer must be supervised with care. Cast, extruded, injection, and compression molded stock all have different working properties.

With the advent of experiments involving

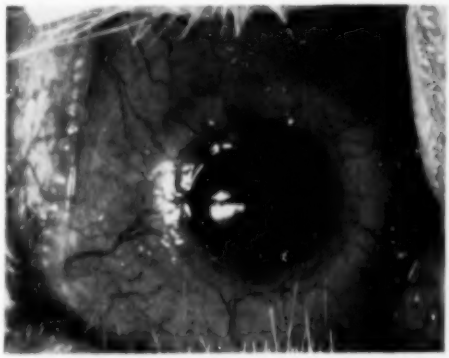


Fig. 6 (Stone). Rabbit 83. Trephine hole in corneal layer anterior to plastic disc has remained patent three months, 12 days. Operative date, March 4, 1954. Photographed June 8, 1954. Disc of 1.5-mm. thickness at center.

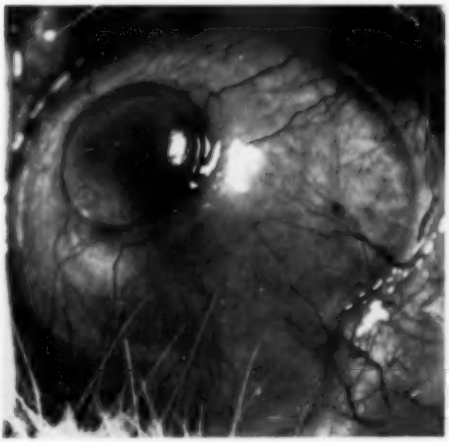


Fig. 5 (Stone). Rabbit 81. Trephine hole in corneal layer anterior to plastic disc has remained patent three months, 14 days, as of June 16, 1954. Operative date, March 2, 1954. Photographed June 8, 1954. Disc of 1.5-mm. thickness at center.

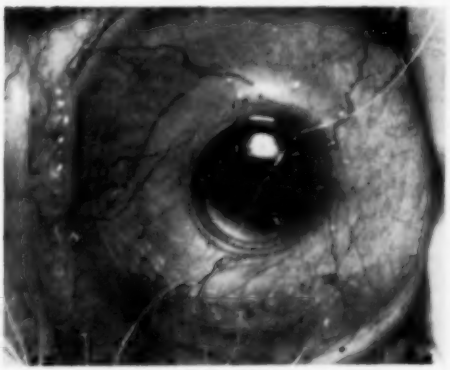


Fig. 7 (Stone). Rabbit 87. Trephine hole in corneal layer anterior to plastic disc has remained patent three months, one day, as of June 16, 1954. Operative date, March 15, 1954. Photographed June 8, 1954. Disc of 1.5-mm. thickness at center.

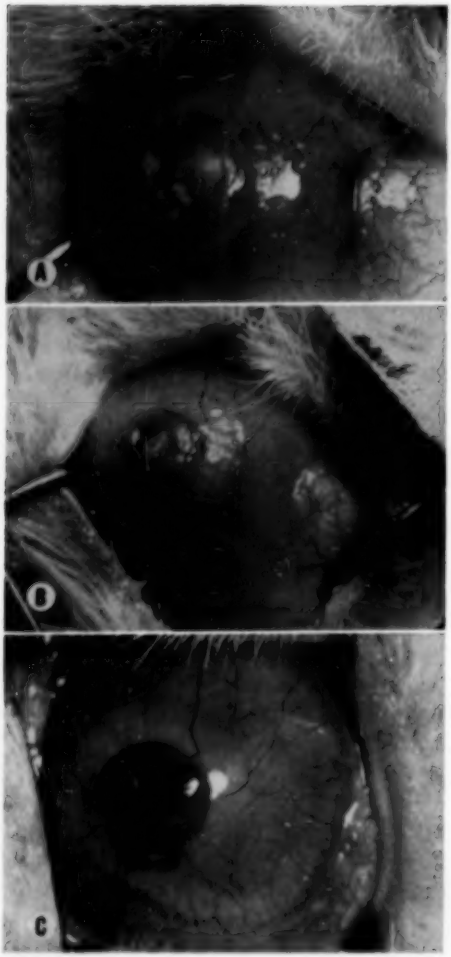


Fig. 8 (Stone). Rabbit 82. Trephine hole in corneal layer anterior to plastic disc shown closing. Disc of 1.5-mm. thickness at center. (A) Two months, three days postoperative. Half-moon-shaped, salmon-colored tissue encroaching on hole from one side. (B) Two months, 21 days postoperative; opening is smaller. (C) Three months, four days postoperative; opening has closed completely.

thickness of the plastic, absorption and porosity properties of varying batches of plastic may have to be tested. To evaluate the role which the shape of the disc plays in relation to thickness, it will be necessary to control molding procedures rigidly. It seems possible that small changes in the shape of the anterior central area of the disc may be of importance.

Discs must be individually molded in the periphery to different rabbit eyes, for the weight of the rabbit and the breed produce great variation in the size and convexity of the cornea.

Because of these and other variables, it has been found that the preparation and handling of the plastic discs is of equal, if not greater importance, in this work than the animal operative procedures.

Discussion and conclusions

Openings have been maintained in the anterior corneal layer in apposition to interlamellar plastic discs in several rabbit eyes for considerably longer than the previously

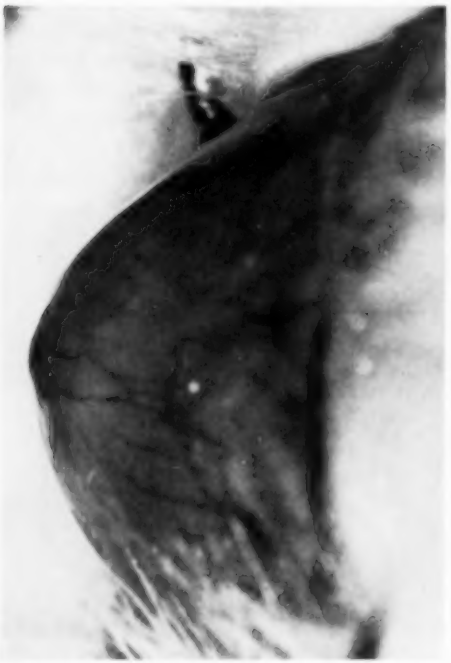
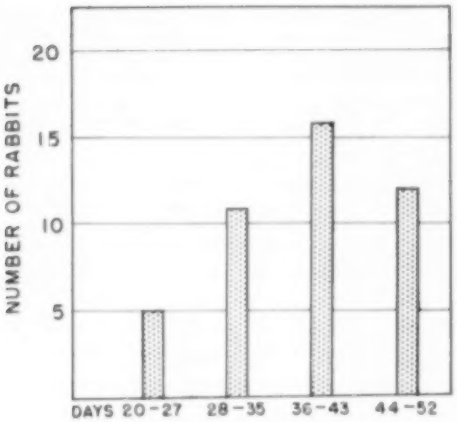


Fig. 9 (Stone). Rabbit 64. Trephine hole in corneal layer anterior to plastic disc closed over in 38 days. Plastic disc, 0.25-mm. thickness throughout, same shape as 1.5-mm. thickness discs.



Graph 1 (Stone). Time of closure of trephine holes in corneal layer anterior to plastic disc with thickness of 0.25 mm.

established maximum time (graph 1). This is with the one exception of the rabbit reported in the previous paper[1] in 1952. At that time there was no indication as to why the opening remained patent. At present it seems that either the shape or the thickness of the plastic disc is the controlling factor. Study is underway in this laboratory to determine which one of the two factors may be the more important.

The related problem of why a specific fabrication of these discs inhibits corneal growth is also being investigated. The relation of porosity and absorption properties of the plastic to nutritional or other factors from the anterior chamber is being considered. We hope we may utilize this procedure of plastic disc implantation as a method to study general factors influencing tissue growth.

Many problems remain to be solved before human experimentation should be attempted. Premature efforts in this direction might well work to the detriment of the procedure and to the individual volunteer.

It must be emphasized that the fabrication of these elements should remain in the hospital laboratory so that their production may be critically supervised and so that the possibility of overenthusiastic exploitation may be minimized.

243 Charles Street (14).

Reference

1. Stone, W., Jr., and Herbert, E.: Experimental study of plastic material as replacement for the cornea, a preliminary report. Am. J. Ophth., **36**:168-173 (June, Pt. II) 1953.

Discussion

Dr. Warren S. Reese: I think Dr. Stone is to be congratulated on having pioneered such an intriguing subject as corneal implants.

Several of us at the Wills Eye Hospital have partly duplicated these experiments, and we presently have a number of rabbits in which these implants have remained for as long as one year. We have not succeeded in maintaining an implant in a cornea that has been trephined, and I believe this coincides with Dr. Stone's experience.

I would like to ask Dr. Stone whether he has done any of these experiments on human enucleated eyes. I suspect that the technical difficulties might be less on human eyes than on rabbit eyes.

I should also like to ask him whether he routinely uses any drugs, such as antibiotics, and so on, in these experimental animals.

Dr. Lorand V. Johnson: I have been intrigued with the potentialities that these studies have presented. Likewise, I am curious about the reaction of tissue around foreign matter in the cornea. I wish Dr. Stone would clarify a little more his thinking on the shape of the disc and its relation to extrusion.

Dr. William Stone, Jr., (closing): I should like to thank Dr. Reese and Dr. Johnson for their remarks.

I think that possibly I did not make a point sufficiently clear, for we have definitely maintained a number of implants in rabbit corneas which have been trephined. The longest one with a trephine has remained in place, as of the present, a period of eight months. The longest interval we have maintained an implant without a trephine is three years, three months, until the natural death of the animal.

In answer to Dr. Reese's question about human experimentation, we have been working on this problem for about five years, and we are firmly of the philosophy that until everything has been done that can be done on laboratory animals, it would be unfair to human volunteers and also unfair to the procedure to attempt human experimentation. If a

person has been blind for a number of years, certainly he can continue for a few more years until we can be more certain that this experiment will help him when it is used.

I am very much against human experimentation at this time. I think it would be premature, and I certainly would not do it myself.

As far as antibiotics are concerned, we carry on a completely sterile technique with our rabbits, and we use antibiotics and atropine every day for a week after the operation; otherwise, we find that our morbidity through infection is very high.

In reply to Dr. Johnson's question, I would like to show another slide.

(Slide) As far as the shape of the implant is concerned, these are the different series and shapes of implants that we have used. We find that small differences in shape make a tremendous difference insofar as whether the implant remains in place or not. We find that practically each individual rabbit has to have an individual implant made for it; because different species of rabbits and different weights of rabbits vary considerably in the size of their corneas and in the convexities of their corneas. We cannot make a definitive statement as to whether it is thickness or shape of the plastic at present which maintains patency.

AN UNSUCCESSFUL ATTEMPT TO PRODUCE HYPERSENSITIVITY TO UVEAL TISSUE IN GUINEA PIGS*

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I. INTRODUCTION

It has long been known that various apparently unrelated substances have an adjuvant effect upon the development of hypersensitivity to antigens in experimental animals. This interesting subject has been reviewed by Freund.[1]

Clark, Zellmer, and Stone[2] found that the production of agglutinins for B. typhosus was increased in rabbits by the previous administration of heat-killed gram-positive cocci.

Lewis and Loomis[3] showed that the capacity of guinea pigs to react to antigenic substances by antibody formation was increased by concomitant tuberculous infection. They further showed that dead tubercle bacilli, trypan blue, and injection of Bacillus abortus or streptococci had similar adjuvant effects.

These observations were confirmed and extended by Dienes[4] and Dienes and Schoenheit.[5] They found that both anaphylactic and tuberculin type hypersensitivity to horse serum or egg-white antigen were produced when these antigens were injected into subcutaneous tuberculous lesions.

Burky[6] found that staphylococcus toxin had an enhancing effect on the development of hypersensitivity to poorly antigenic substances. Coulaud[7] found that sensitization to tuberculin in guinea pigs developed to an unusually high degree when killed tubercle bacilli incorporated in paraffin were injected subcutaneously. Ramon, Lemetayer, and Richou[8] found that paraffin oil or lanolin both enhanced the formation of diphtheria antitoxin.

Freund and McDermott[9] reported the adjuvant effect upon development of hypersensitivity by combining an aqueous solution of antigen with paraffin oil containing killed tubercle bacilli, using as an emulsifying agent, aquaphor, and an ointment base derived from lanolin. Freund's report stimulated widespread interest and an enhanced immune response to a variety of substances was obtained by various workers[10] using Freund's technique.

Kabat, Wolf, and Bezer[11] and Morgan[12] produced an acute disseminated encephalomyelitis in monkeys by intramuscular injection of central nervous system tissue with adjuvants. Kabat, Wolf and Bezer[13] performed right frontal lobectomies on six rhesus monkeys and froze the removed brain

* From the Wilmer Ophthalmological Institute of The Johns Hopkins Hospital and University.

tissue. Following recovery, each monkey was given three intramuscular injections, at weekly intervals, of emulsion of its own brain using Freund's technique. Five of the six animals developed symptoms of acute disseminated encephalomyelitis within four to 28 days after the third injection.

Freund, Stern, and Pisani[14] reported an isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and mycobacteria in water-in-oil emulsion.

Lucic[15] reported on the sensitization of rabbits to uveal tissue by the synergic action of staphylococcus toxin. He employed the technique described by Burky in producing lens sensitization, and reported that six of 22 rabbits given repeated weekly injections of bovine uveal pigment and staphylococcus toxin showed sensitization to uveal pigment by histologic examination of skin test site 14 days after testing.

Collins,[16] in 1949, reported the production of large areas of focal infiltration with lymphocytes and epithelioid cells in the choroid of 12 of 25 guinea pigs given macerated whole guinea pig uvea using the adjuvant technique of Freund. Nineteen control animals given either adjuvant alone or adjuvant plus an unrelated tissue, liver, showed no abnormal histopathologic findings. Seventeen of the 25 animals were tested intradermally with uveal pigment, and showed no tuberculin-like reaction. Quantitative complement fixation tests to whole guinea pig uvea were done on 12 animals and found to be negative.

II. Experimental procedure

Following the publication of Collins' report, it appeared worthwhile to extend his work, using uveal antigen from both albino and nonalbino guinea pigs. The following experiment was therefore carried out.

Uveal tissue antigen was obtained from healthy guinea pigs, albino and nonalbino, which had been stunned and exsanguinated. Immediately after exsanguination, the eyes were enucleated and frozen in carbon dioxide snow. The eyes were later thawed out, dipped for several seconds in boiling water, and the entire uveal tract removed under sterile technique. The uveal tracts were ground in sterile saline, in a mortar, and then further ground in a tissue grinder. Normal saline was added so that one uveal tract was suspended in 0.5 cc. of saline. Phenol was added to make a 0.5-percent solution. The uveal tissue suspensions were placed in sterile rubber stoppered bottles. Separate suspensions were obtained with albino and nonalbino uveas.

Lens antigen was obtained from the same eyes as the uveal tissue antigen. The lens capsule, with adherent uveal pigment, was removed and the remaining lens tissue was ground in sterile normal saline in a motar. Normal saline was added so that one lens was suspended in 0.5 cc of saline. Phenol was added to make a 0.5-percent solution and the suspension was placed in sterile rubber stoppered bottles.

Four water-in-oil emulsions were prepared according to the technique of Freund, using pigmented uveal antigen, albino uveal antigen, lens antigen, and 0.5-percent phenol in saline. One part of autoclaved aquaphor was added slowly to two parts of antigen in saline in a sterile mortar, and mixed thoroughly. One part of autoclaved paraffin oil, in which had been suspended 2.0 mg. (dried weight) of heat killed virulent human tubercle bacilli per cc., was then added. One cc. of final emulsion therefore contained one uveal tract or one lens and 0.5 mg. of tubercle bacilli, 0.5 cc. of saline (plus phenol), and 0.25 cc. of aquaphor, and 0.25 cc. of paraffn oil.

Seventy-two guinea pigs, male and female, albino and nonalbino, weighing between 250 and 750 gm. were divided into six groups of 12 animals and each animal was given two subcutaneous injections, one week apart, of 1.0 cc. of emulsion as follows:

Group I. Pigmented uvea in water-in-oil emulsion.

Group II. Albino uvea in water-in-oil emulsion.

Group III. 0.5-percent phenol in saline in water-in-oil emulsion.

Group IV. Lens tissue in water-in-oil emulsion.

Group V. Pigmented uvea in saline.

Group VI. Albino uvea in saline.

The animals were examined with a hand-light and ophthalmoscope every two to three days. Half of the animals in each group were killed five weeks after the first injection, and the remainder seven weeks after the first injection.

Two weeks before death, each animal was given an intradermal skin test consisting of 0.1 cc. of guinea pig uveal tissue suspension in saline, both albino and nonalbino, as prepared previously. Forty-eight hours before death, each animal was given a series of intradermal skin tests which included (1) 0.1 cc. of old tuberculin (0.1 mg./cc.), (2) 0.1 cc. of guinea pig lens in saline, (3) control. All skin tests were observed at 24 and 48 hours, and the uveal skin tests injected two weeks previously were excised at time of death. At the time of death all eyes were enucleated and subsequently examined histologically.

III. Results

The results of the various skin tests as observed 48 hours after injection are shown in Table 1. In no case was a tuberculin-type skin reaction noted to either pigmented uvea or to nonpigmented uvea. That the animals were capable of developing tuberculin-type skin reactions is shown by the uniformly strong reaction to both old tuberculin and to lens protein.

The excised pieces of skin containing the uveal skin tests were examined histologically. In no case was a positive or even doubtful reaction observed.

The enucleated eyes of all animals were examined histologically after staining with hematoxylin and eosin. The only abnormalities noted were occasional scattered small collections of lymphocytes in the uveal tracts. No epithelioid cells were found, and no lesions resembling Dalen-Fuchs nodules were found. The scattered collections of small lymphocytes were found in approximately one third of all the eyes, and were found in approximately equal numbers in all six groups.

IV. Comment

Freund's adjuvant technique has proven itself a powerful tool in the experimental production of hypersensitivity to various proteins. The work of Collins suggested that the application of this technique offered an excellent method for the study of hypersensitivity to uveal tissue in experimental animals. Under the conditions of this single experiment, Collins' results were not confirmed. It is difficult to be certain whether this was

TABLE 1

Results of intradermal skin tests as observed 48 hours after injection

Group	Pigmented Uvea	Albino Uvea	Old Tuberculin (0.1 mg./cc.)	Lens	Control
I. Pigmented uvea in water-in-oil emulsion	0	0	+++	0	0
II. Albino uvea in water-in-oil emulsion	0	0	+++	0	0
III. 0.5% phenol in saline in water-in-oil emulsion	0	0	+++	0	0
IV. Lens tissue in water-in-oil emulsion	0	0	+++	+++	0
V. Pigmented uvea in saline	0	0	0	0	0
VI. Albino uvea in saline	0	0	0	0	0

the result of some unknown factor in the technique here used or whether the proteins of the uveal tract are unusually reluctant in the production of a hypersensitive state. The fact that a tuberculin-type skin sensitivity to lens protein was produced suggests that the latter explanation may be the correct one.

V. Conclusions

Using Freund's adjuvant technique, an attempt was made to produce hypersensitivity to uveal tissue in guinea pigs. Under the conditions of the experiment neither a tuberculin-type skin hypersensitivity to uveal tissue nor a positive uveal pigment skin test as determined histologically could be produced. Likewise, there were no histologic lesions produced in the eyes that were in any way suggestive of sympathetic ophthalmia. Hypersensitivity to lens protein as demonstrated by tuberculin-type sensitivity was produced using the adjuvant technique.

The Johns Hopkins Hospital (5).

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FILTER-PAPER ELECTROPHORESIS OF TEARS*

I. Lysozyme and its correlation with keratoconjunctivitis sicca

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It has been difficult heretofore to study the protein components of tears because of the minute amount of material available. The simplicity and micro character of filter-paper electrophoresis[1] allows the investigation of the proteins in the fluid in the conjunctival sac and provides a new avenue of approach to the understanding of pathologic conditions of the conjunctiva. This paper reports the application of such a method and the results so far obtained in the study of normal and pathologic conditions of the eye.

Apparatus

A 12 by 25 cm. sheet of Whatman No. 1 filter paper is placed on a plastic rack in such a fashion that it is supported longitudinally about every 2.0 cm. for a distance of 20 cm., leaving 2.5 cm. on each end to dip into a buffer solution. The buffer vessels contain about 150 ml. of a barbital—NaCl buffer (pH = 7.8, $\mu = 0.16$) and Ag—AgCl electrodes to which is applied a direct current of 70 volts to give a gradient 3.5 v./cm. and a current of about 6 ma. These component parts are totally enclosed in a plastic box to provide an atmosphere of constant humidity.

Method

The tear fluid from the conjunctival sac is absorbed on 0.25-inch Φ Whatman No. 1 filter-paper disc which is then placed in a small constant humidity chamber to keep it moist until used. The specimen will be good for several days although it is preferably analyzed the same day. The electrophoresis apparatus is assembled, equilibrated for a short period of time, and the specimen is placed on the filter paper about 7.0 cm. from the negative[2] electrode. Four specimens may be run at the same time including a standard consisting of a solution of crystalline egg white lysozyme (0.25 percent) and of albumin[†] (0.4 percent). The current is turned on and allowed to run about 16 hours. The filter paper sheet is removed, air dried, heated 10 minutes at 105°C. and stained in the usual manner[1] with brom phenol blue and the blue color developed with ammonia.

Normal patterns

Under the above conditions, the normal pattern consists of at least three components. One component travels to the negative pole with an electrophoretic mobility of 2×10^{-5} cm./sec./volt/cm. (uncorrected for endosmosis). This component, as will be shown later, is the lysozyme component. Another component moves to the positive pole with a mobility of 3.5×10^{-5} cm./sec./volt/cm. This is the fastest moving component and seems identical with albumin. The third component is probably an undifferentiated mixture of proteins which will be analyzed under different electrophoretic conditions in

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† Purified Cohn fraction V, courtesy of Cutter Laboratories, Berkeley, California.

order to obtain good separation and identification. The mobilities of both the lysozyme and the albumin are slower than those values found for liquid electrophoresis[3, 4] which indicates that the proteins are not traveling solely in the buffer film.

In the normal individual, regardless of sex or age, whose tears have been analyzed, this pattern is reproducible. Tears from crying individuals whether emotionally or chemically produced for short durations give the same pattern. Repeated analyses of tears from 20 cases of chronic catarrhal conjunctivitis also show this normal pattern.

Abnormal patterns

It is interesting that the only deviation we have found from the normal pattern is either a lack of the lysozyme component or a greatly reduced concentration of it. This deviation has been found in the following conditions. Six cases of clinically definite keratoconjunctivitis sicca were studied and were found to be lacking in the lysozyme component.[5] Two cases were found which had symptoms and complaints compatible with either keratoconjunctivitis sicca or chronic catarrhal conjunctivitis. Since they had a greatly lowered lysozyme concentration and showed negative cultures it is felt that these cases represent an early keratitis sicca. There was one case of a unilateral dry eye secondary to trachoma which lacked the lysozyme component. This was presumed to be because of fibrosis of the lacrimal gland as a result of an adenitis and the scarring of the conjunctiva.

Discussion

In view of the above correlation of lack of the lysozyme component with keratoconjunctivitis sicca and the possible detection of early cases it became necessary to show that the negatively moving component is lysozyme beyond the evidence that it is electrophoretically indistinguishable from crystalline egg white lysozyme. This is important also in view of the report of Smollens,[6] et al., who indicate that the lysozyme of tears is different from the lysozyme of egg white.

An electrophoretic run was carried out on two normal tears, two sicca tears, and one 0.25-percent egg white lysozyme solution. The egg white lysozyme strip and one each of the normal and sicca strips were cut into 0.75-inch sections on either side of the origin. These sections were placed in buffered solutions (pH 6.8) containing the polysaccharide coat of Micrococcus lysodeikticus* following a modification of the method of Salton.[7] The reduction in optical density at 640 mμ indicates the clearing of mixture by hydrolysis of the polysaccharide. By definition[8] this shows the presence of lysozyme. Table 1 gives the results of such an experiment and shows that only egg white lysozyme and normal tears have lytic activity and this activity is confined to the negatively moving component. The unused strips of normal and keratitis sicca tears were stained and the latter strip showed no lysozyme component while the position of the lysozyme com-

* Obtained through the courtesy of the Department of Medicine.

TABLE 1

Effect of serial sections of different electrophoretic diagrams on Micrococcus lysodeikticus as a test for lysozyme

(Optical density at 640 mμ)

Material	Section*						
	1	2	3	4	5	6	7
Lysozyme control	0.93	0.56	0.94	0.92	1.00	0.98	1.02
Normal	0.94	0.55	0.77	0.98	1.00	0.99	1.00
Keratitis sicca	0.98	0.96	0.98	0.99	0.98	0.99	0.98

* Sections numbered from negative pole to positive pole with the origin between Sections 3 and 4.

ponent of normal tears was shown to correspond with the position of the activity of the enzyme toward M. lysodeikticus.

It would thus appear that the lysozyme of human tears is electrophoretically and enzymatically similar to egg white lysozyme and is quite distinct from the other proteins of tears.

Summary

A filter paper electrophoretic method for the protein analysis of an individual's tear fluid has been described. One protein component moves to the negative pole at pH 7.8 and has been identified as the lysozyme fraction by its electrophoretic mobility and by its activity toward M. lysodeikticus. There is a direct correlation between the lack of this component and the presence of keratoconjunctivitis sicca. There is an indication that it is possible to detect early cases. In the cases studied there has been no other pathologic condition that has shown this defect except for a dry eye secondary to scarring in trachoma.

University of California Medical Center (22).

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Discussion

Dr. Harry Green: In 1949, Smollens, Leopold, and Barker conducted electrophoretic studies of the component fractions of tears derived from human patients. At that time with an apparatus not adapted for micro-work, they found essentially four components, three of which migrated to the cathode, the negative electrode, and one migrated to the positive electrode. They assumed that the lysozyme was contained in the three components that migrated to the anode.

Dr. Leopold, who regrets he was unable to attend this meeting to discuss this interesting paper, has informed me that most recently he and Dr. Smollens have conducted similar experiments with a micro-electrophoretic apparatus, and they have again found four components. However, the one component that contained lysozyme fraction now migrated to the cathode, the negative electrode, and the three inactive components migrated to the anode. This is essentially in agreement with the results presented today.

Caselli and Schumacher (Klin. Monatsbl. f. Augenh., **124**:148-154, 1954) have reported the same results—four components, three of which migrated to the anode, and the component containing lysozyme migrated to the cathode.

OCULAR LESIONS IN HAMSTERS*

With chronic *TOXOPLASMA* and *BESNOITIA* infection

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Uveitis has been produced experimentally, usually in rabbits, by injecting tubercle bacilli or other micro-organisms directly into the anterior chamber.[1, 2] Hogan[3] described the production of acute chorioretinitis by the intracarotid injection of rabbits with Toxoplasma.

The purpose of this report is to describe the observation of eye lesions incidental to a study of two generalized infections, induced by subcutaneous or intraperitoneal inoculation in golden hamsters (Mesocricetus auratus). It is felt that such infections represent better models for comparison with sporadic chorioretinitis in man.

Localization in the eye, development, and extension of lesions can be studied in proper perspective to the infection elsewhere in the host. Lesions produced by proliferating Toxoplasma gondii and Besnoitia jellisoni organisms, or by the rupture of their cysts, can be compared.

Immunologic factors can be investigated that may allow eventually a better understanding of the pathogenesis of infections giving rise to lesions in various tissues of the body as well as in the eye.

To introduce the ocular pathology, a review of the pathogenesis of each infection in the hamster will be given.

Pathogenesis of toxoplasmosis in the hamster

This subject has previously been considered in greater detail.[4, 5] The following data are relevant to this study. Proliferative forms (fig. 1 and 4) and cysts (fig. 5 and 8) of Toxoplasma are recognized. Distinctive features are the argyrophilic and periodic acid-Schiff (PAS) positive cyst wall that is resilient in the fresh state. Individual Toxoplasma within cysts are generally more densely packed, more slender, and possess a large paranuclear glycogen granule, whereas proliferative organisms contain several smaller glycogen granules that are scattered.

When hamsters are inoculated with either form subcutaneously on the back, or intraperitoneally, a generalized infection develops, with lesions predominantly at the injection site, in the lungs, liver, and spleen. Untreated hamsters generally succumb to the acute infection within five to 15 days, depending on size of inoculum, the virulence of the strain, and other factors.[5] Examination of tissue sections shows the presence of large numbers of proliferative forms of Toxoplasma, intracellularly as well as extracellularly, producing lesions by necrosis of parasitized cells and accompanying inflammation.

When hamsters are treated with sulfadiazine, the acute infection may remain more or less asymptomatic. Within two to four weeks hamsters have generally acquired sufficient immunity so that upon discontinuation of therapy, they survive with little or no symptoms after an infection with most strains of Toxoplasma. When examined by subinoculation, organisms can generally be isolated from the brain and from some other organs.[5] On tissue sections, cysts of Toxoplasma are found in small numbers only, usually free in the brain without accompanying tissue reaction. Organisms or lesions are rarely encountered in the eyes.

Chronic toxoplasmosis induced by a majority of laboratory strains is a latent infection in most laboratory animals. However, infection with the RH strain[6] in golden ham-

* From the Department of Pathology and Oncology, University of Kansas School of Medicine. This investigation was supported in part by a research grant E-547 from the National Microbiological Institute of the National Institutes of Health, Public Health Service.

sters results in a chronic infection that is frequently accompanied by symptoms of encephalitis, ocular involvement, and adrenal necrosis. These have been described elsewhere.[5] It is sufficient to state here that these symptoms appear weeks and months after infection at a time when high antibody titers are present.

In the brain numerous Toxoplasma cysts are found as well as foci of necrosis and glial nodules. The intact cysts are not chemotactic, and the lesions result from cyst rupture; there is no histologic evidence of toxoplasmic proliferation. The adrenal lesions, however, are produced by proliferative organisms. Hence, there is evidence that cysts as well as proliferative organisms persist during chronicity. Discussion of the eye lesions will be found later.

Pathogenesis of infection with Besnoitia in the hamster

Besnoitia jellisoni has recently been described as a protozoan resembling Toxoplasma.[7] Proliferative forms (figs. 11 and 14) and cyst forms (figs. 19 and 22) are recognized. Inoculation of hamsters with either form leads to an acute infection in which only proliferative forms are found. After subcutaneous inoculation, a generalized infection develops with lesions predominantly at the injection site, in the lungs, liver, spleen, and adrenal gland, much like that seen in toxoplasmosis. Untreated hamsters die with their acute infection between the eighth and 12th day. Tissue sections show lesions related to the parasitism of individual cells. Intracellular and extracellular proliferative forms of Besnoitia together with necrosis of host cells and inflammation are present.

Treatment of infected hamsters with sulfadiazine (60 to 90 mg. percent of the sodium salt in the drinking water) retards the proliferation of organisms sufficiently to prevent death. It also permits the development within 10 to 14 days of a state of relative immunity.

In the third and fourth weeks of infection, cysts make their appearance at the injection site and elsewhere. Dependent on their state of development they range from 20 to 500 micra in diameter and consist of a resilient cyst wall, five to 50 micra in thickness, enclosing a syncytium of large vesicular nuclei and a cytoplasmic vacuole in which the crescentic Toxoplasma-like organisms develop (fig. 19).

In the larger cysts, a single vacuole filled with micro-organisms occupies nearly the entire cyst, with the large vesicular nuclei inconspicuously applied to the cyst wall (fig. 22). These cysts are not accompanied by a significant inflammatory reaction as long as they are intact (fig. 18).

Chronic Besnoitia infection is ordinarily accompanied by the presence of cysts. The lesions following the breakdown of cysts are characterized by necrosis, infiltration of granulocytes, and later mononuclear cells and fibrosis (fig. 24). The organisms liberated by cyst rupture are found to be poorly staining and subject to phagocytosis. The lesions are circumscribed, resembling a granuloma, and there is no evidence that Besnoitia liberated from cysts enter new cells and proliferate.

Proliferative forms of Besnoitia, however, do survive into the chronic stage of infection. This can be easily demonstrated by the use of certain strains that have lost their cyst-forming capacity, and where only proliferative forms occur. Such strains have been employed during this study to follow the more evenly progressing infection with proliferative forms. This avoids the complication resulting from cyst rupture with the release of large amounts of apparently non-infective antigenic material. Conversely, lesions produced by cyst rupture uncomplicated by marked proliferative activity can be studied when the animals are maintained on sulfonamide therapy which inhibits proliferative organisms to a greater extent than the development of cysts and contained organisms.

Experimental procedures

Hamsters were infected by the subcutaneous or intraperitoneal route with either Besnoitia or Toxoplasma. They were then treated with sulfadiazine-sodium, in doses of 60 to 120 mg. percent in the drinking water. Treatment was usually begun on the day of inoculation and was continued for 10 to 30 days, and occasionally intermittently. To control the infection with the RH strain of Toxoplasma, it was often necessary to incorporate additional sulfadiazine into the food.

Certain of the hamsters were first infected with one of several other strains of Toxoplasma (without producing eye lesions). They were then secondarily infected with the RH strain, following which ocular lesions did appear.

Eye examination

Hamsters were periodically examined for the development of eye lesions. After they had been placed in the dark for one to two hours, or at night, their pupils were widely dilated so that no mydriatic was necessary.

The animal to be examined is picked up by the loose skin over the back of the neck and, occasionally, if skin tension closes the eye, or, if the hamster resists handling, it is held by placing thumb and first finger of the left hand under its chin from posteriorly.

The optic reflex is first observed by shining a light from close and somewhat laterally into the hamster's eye, watching from some distance. In the normal eye a bright reflex is obtained from a dark fundus, with the media appearing clear. Following this, with the plus-eight lens of the ophthalmoscope applied closely to the observer's eye, the hamster's eye is approached to about five cm. and the fundus is visualized.

On a yellowish or pinkish background, red vessels are seen converging toward the optic disc, which usually projects into the vitreous and generally shows black pigmentation. Minor dioptric adjustments are made, and the fundus is scanned quickly. This is often facilitated by use of a green filter which reduces the glare from the light fundus.

Although hamsters occasionally attempt to bite when handled while in the light, they did not attempt to do so when handled in the dark. Only animals with encephalitis need to be handled with care.

Although most hamsters struggle slightly, examination of the eye is an easy procedure, especially if the animals are examined once or twice weekly. The plus-20 lens is more useful for examination of the anterior chamber, iris, and lens, since the plus-40 lens requires approaching the animal too closely, stimulating its vibrissae and making it restless.

With Besnoitia infection, eye lesions appeared in over 50 percent of animals during the first two months. Hence, more of the eyes came to study during the early stages of their involvement.

With Toxoplasma infection, eye lesions appeared sporadically and often months after infection. Since at that time animals were under less close observation, the earliest lesions were frequently missed. Many of the involved eyes were observed incidental to experiments in which it was not feasible to kill the animal. In such instances, enucleations were performed. In all other cases the entire animal was studied histologically.

The eyes were usually fixed in Zenker-formol solution for eight to 10 hours and embedded in methyl benzoate containing one-percent celloidin followed by paraffin. Sections were stained with hematoxylin and eosin or with the periodic acid-Schiff technique followed by hematoxylin (PASH). Selected eyes were serially sectioned, or semiserially, by mounting six sections, five micra in thickness, every 100 micra. The eyes of 35 hamsters showing clinically observed lesions during Besnoitia infection form the basis for this histologic study.

A similar number of eyes from animals with chronic toxoplasmosis were available,

but nine eyes with representative lesions were selected for detailed histologic study based on the availability of semiserial sections. Hamster eyes measure approximately four to five mm. in diameter after fixation and sectioning, with the lens accounting for over half of this diameter.

Ocular lesions associated with chronic toxoplasmosis

Incidence

Due to the sporadic and late appearance of lesions no exact data are available. Unexplained factors account for some variability, since eye lesions were observed in an estimated 60 percent of hamsters several years ago, associated with an incidence of about 80 percent of encephalitis, whereas during the last two years the incidence of each has been in the neighborhood of only 40 percent. Clinical data on individual hamsters have been reported.[5]

Widespread retinal necrosis

This lesion was seen in two eyes from two hamsters. One (No. 361) had been infected with the RH strain for three months and it had been treated with sulfonamides for the first three weeks after infection. The other hamster (No. 315) had been infected with the CJ strain of Toxoplasma[8] for one year, and had been treated during the first month of the infection; it was reinjected with CJ after two and five months, and was finally challenged with the RH strain 10 months after initial infection. During the next two months, it lost weight, developed signs of encephalitis, and became paraplegic.

Destruction of the retina was most severe at the posterior pole. In one of the eyes the more anterior parts of the retina were little involved, but it was detached by exudate from the choroid. Neutrophil granulocytes were most common in and near the necrotic

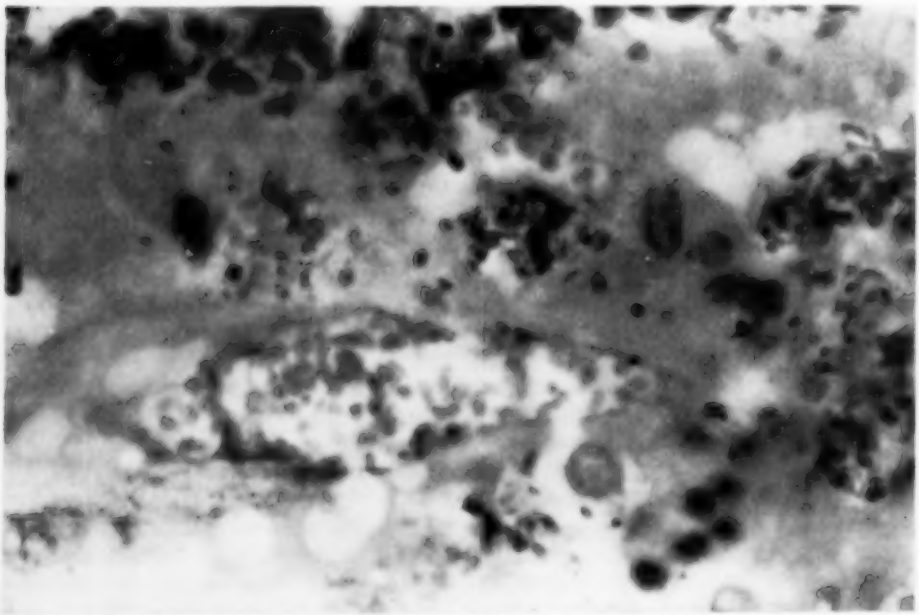


Fig. 1 (Frenkel). Proliferative forms of Toxoplasma in retinal pigment epithelium. Most of the central retina is necrotic; lymphocytes and mononuclear cells are present in the adjacent choroid. (Hamster 315. ×1200. Periodic acid-Schiff followed by hematoxylin.)

portions. Most of the pigment epithelium was destroyed with much dispersal of pigment granules.

Proliferative organisms of Toxoplasma were identified in the retina only (fig. 1). The choroid showed a predominance of lymphocytes in one and plasma cells in the other eye, without necrosis. The ciliary body pigment was slightly dispersed and the stroma was infiltrated with lymphocytes, but the epithelium was intact. The iris contained a few plasma cells; some pigment-laden cells were present in the anterior chamber. The optic nerve, sectioned only in one eye, was necrotic; the scleras showed little change. The contralateral eyes of both hamsters were subinoculated into mice and Toxoplasma was isolated from both.

Focal chorioretinitis associated with proliferative Toxoplasma

This was seen only once in an otherwise normal appearing hamster (J54-59) infected with the RH strain via the subcutaneous route. It had been treated with sulfadiazine for 17 days, was challenged with RH, and two days later treated for another eight days. Between nine and 10.5 months after infection a small number of pigmented cells appeared in the anterior chamber of one eye and it was difficult to visualize the fundus. The eye was enucleated and sectioned semiserially.

Only a single focal chorioretinal lesion was found (fig. 2) measuring 1.4 mm. by 0.6 mm. in diameters. It was situated about midway between the nervehead and the ciliary body. In the center of the lesion the retina was partially necrotic and moderate numbers of neutrophil granulocytes were present (fig. 3).

The inner and outer nuclear layers were most markedly destroyed and there was dispersal of pigment. The covering ganglionic layer and the entire retina peripherally showed edema, infiltration with cells resembling granulocytes, swollen microglia and lymphocytes. The retinal capillaries were

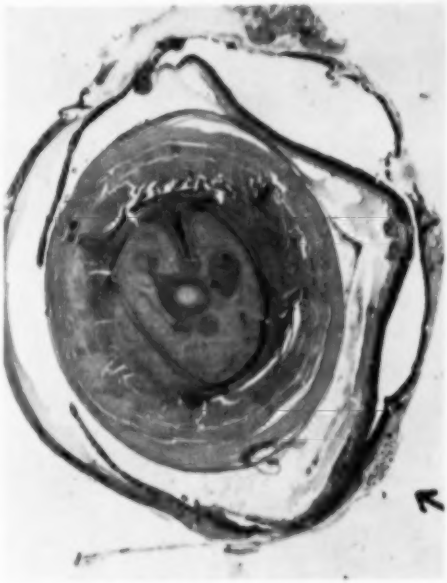


Fig. 2 (Frenkel). Focal chorioretinitis 10.5 months after infection with Toxoplasma. (Hamster J-54-59. ×15. Periodic acid-Schiff followed by hematoxylin.) Figures 3 and 4 are from the same eye.

dilated and surrounded by inflammatory cells.

Small groups of proliferative forms of Toxoplasma were common in vacuoles presumably but not clearly intracellular in position (fig. 4). Up to six groups were observed in one section, the largest containing in the neighborhood of 40 organisms, as counted in serial sections.

Bruch's membrane appeared intact; however, in the center of the lesion fibroblastlike (retinal epithelial?) cells were proliferating. The choroid joining the retinal lesion showed fibroblastic proliferation and dispersion of pigment, and, more peripherally, infiltration with lymphocytes and plasma cells. In the overlying scleras were a few inflammatory cells with pigment.

The vitreous contained microglialike cells, singly and in groups; similar cells were surrounding and wandering away from the vascular cone of the nervehead along the

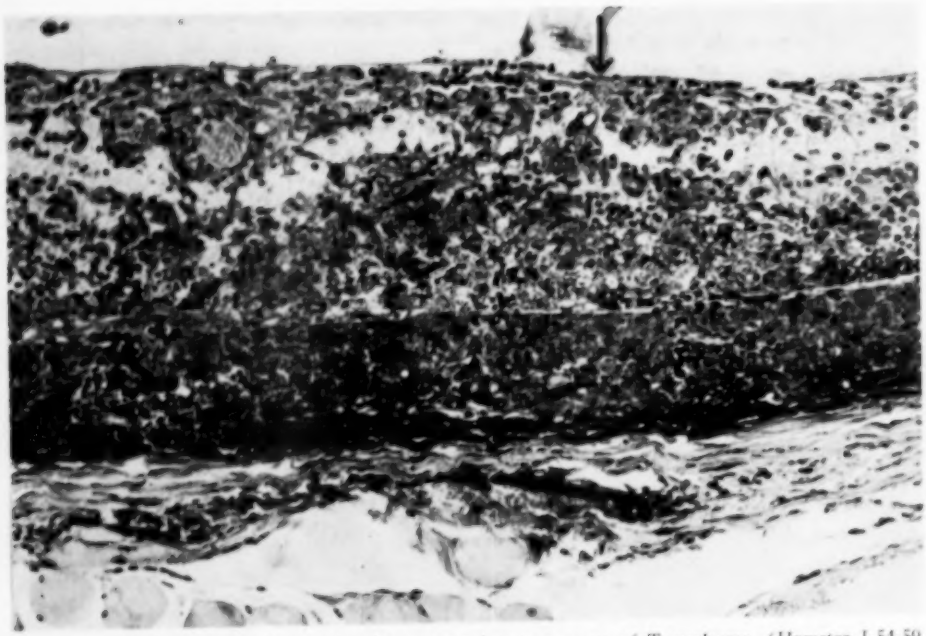
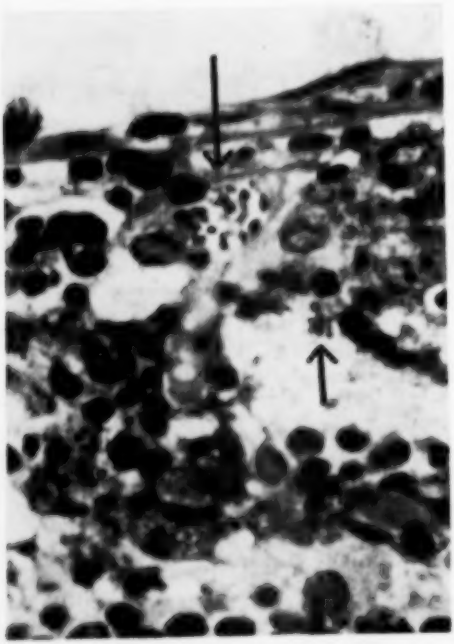


Fig. 3 (Frenkel). Focal chorioretinitis; the arrow points to a group of Toxoplasma. (Hamster J-54-59. ×230. Periodic acid-Schiff followed by hematoxylin.) Figures 2 and 4 are from the same eye.



hyaloid membrane. Retinal vessels, even at a distance, were surrounded by mononuclear cells.

Ciliary body, iris, anterior and posterior chambers contained inflammatory cells. Posterior synechias and pigment-ladened cells covered part of the lens. Cornea and lens appeared normal.

Chorioretinitis associated with Toxoplasma cysts

This was best represented in two hamsters infected with the RH strain for six and seven months. Both had been treated for three weeks following infection.

In Hamster 364 only the left eye was involved; the right fundus could be visualized

←

Fig. 4 (Frenkel). Two groups of proliferative Toxoplasma in retina of hamster J-54-59. The small irregular granules at left are pigment. (×960. Periodic acid-Schiff followed by hematoxylin.) Figures 2 and 3 are from the same eye.

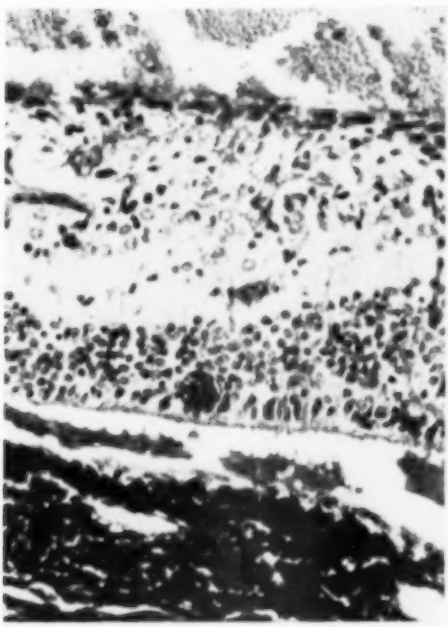


Fig. 5 (Frenkel). Toxoplasma cyst in retina showing chronic inflammation and degeneration of the outer nuclear and the rod and cone layers. "Subretinal" edema is present, but the pigment epithelium remains attached to Bruch's membrane and choroid. There is hemorrhage in the vitreous. (Hamster 386, six months after infection, right eye. Dye test titer 1:65,000. ×230. Periodic acid-Schiff followed by hematoxylin.)

and when sectioned no lesions or cysts were found. A moderate degree of encephalitis was present. Toxoplasma antibody titer as measured by the dye test was 1:65,000.

Hamster 386 had recently developed haziness of the right fundus and cysts were found on sections. The left eye (which will be discussed in the next section) had developed a hemorrhage in the anterior chamber three months after infection, which was followed by keratitis, ulceration, and extrusion of the lens six months after infection. This was treated by the topical application of Sulfamylon (brand of 4-aminoethyl-benzene sufonamide HCl), and a week later the animal was killed. The dye test titer was 1:65,000.

Least advanced changes were seen in the right eye of Hamster 386. The retina showed uniform loss of the outer nuclear layer, with a diffuse gliosis slightly disrupting the inner nuclear and ganglion layers (fig. 5). Lymphocyte and microglialike cells were scattered about.

Toxoplasma cysts were present some of which appeared intracellular in location. They contained 100 to 200 individual organisms which stained brillantly red with the periodic acid-Schiff-technique. Individual cysts extended through several serial sections, and less than one cyst per section was seen.

Hemorrhage was present in part of the vitreous. Much of the latter was obliterated by gliosis joining the retina to the lens. Exudate detached part of the retina from the pigment epithelium (fig. 5). There was only

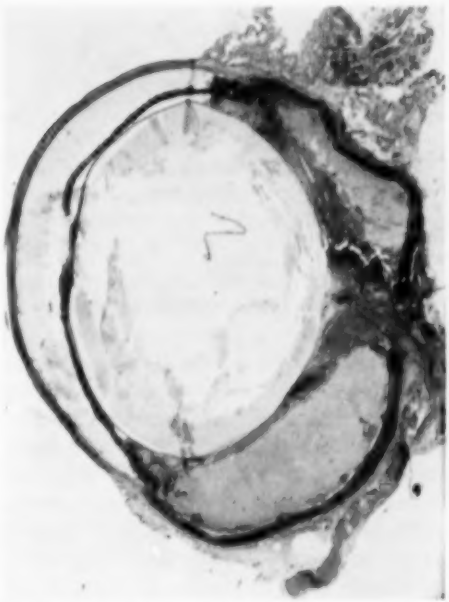


Fig. 6 (Frenkel). Toxoplasmic chorioretinitis associated with cysts. Note formation of inflammatory vesicles and of posterior synechias. (Hamster 364, seven months after infection. Dye test titer 1:65,000. ×15. Periodic acid-Schiff followed by hematoxylin.) Figures 7 and 8 are from the same eye.

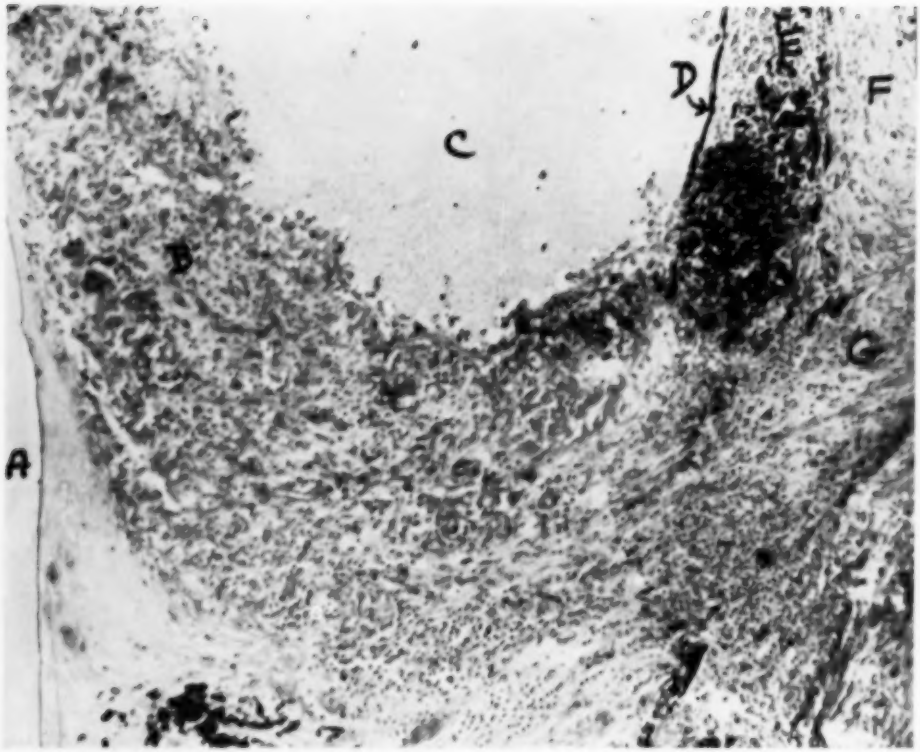


Fig. 7 (Frenkel). Close-up of lesion shown in Figure 6 (sides reversed). (A) Lens which is adherent to retina. (B) Retina showing necrosis, cellular infiltration, fibrin deposits, and cellular proliferation. (C) Inflammatory vesicle containing a few large mononuclear cells. (D) Pigment epithelium. (E) Choroid with chronic inflammation. (F) Sclera. (G) Optic nerve sheath. Only cysts and no proliferative forms of Toxoplasma were identified in this eye. (Hamster 364. ×310. Periodic acid-Schiff followed by hematoxylin.) Figures 6 and 8 are from the same eye.

a moderate degree of choroidal infiltration with lymphocytes and plasma cells. Ciliary body and iris were much enlarged by proliferation of pigmented cells; the epithelia were only slightly disorganized. Pigment-bearing mononuclear cells were present singly and in groups in the anterior chamber as well as on the lens. The pupil was covered by fibroblastic proliferation.

Marked gliosis with inflammatory cells and vascular proliferation, giving the appearance of granulation tissue, together with necrosis of the latter, as well as of most of the retina, were the distinctive features of the left eye of Hamster 364 (figs. 6 and 7).

Toxoplasma cysts were numerous here (fig. 8). Large fluid-filled vesicles were present between the retinal remnants and the pigment epithelium. Mononuclear cells formed small hillocks of cells. These inflammatory vesicles pushed the retinal remnants toward the lens, and the slitlike vitreous remnant was occupied by fibrinous exudate and debris.

Again much proliferation of ciliary body and iris stroma was noted. Some fibroblastic proliferation extended from the ciliary body anteriorly and posteriorly over the lens. Posterior synechias were present together with pigment-ladened inflammatory cells in

the anterior and posterior chambers. Lens and cornea were seemingly unchanged.

Phthisis bulbi

The history of one eye in which lens extrusion occurred was given in the last section. Another eye with less advanced changes came from Hamster *369*, seven months after infection. This latter eye showed shrinkage, degeneration of lens capsule and body, especially posteriorly, where fibrous scar tissue was attached to the lens and showed much pigment and calcification (fig. 9).

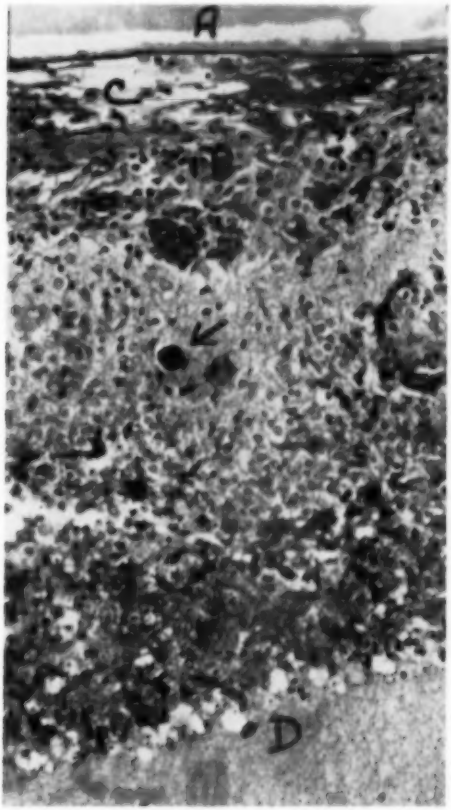


Fig. 8 (Frenkel). Toxoplasma cyst in retina (arrow). Lens (A) and ganglion layer of retina (B) are joined by glial fibers (C). (D) Inflammatory vesicle. (×230. Periodic acid-Schiff followed by hematoxylin.) Figures 6 and 7 are from the same eye.

Fig. 9 (Frenkel). Early phthisis bulbi, seven months after infection with Toxoplasma. (Hamster *369*. ×15. Periodic acid-Schiff followed by hematoxylin.)

The choroid was about five times thicker than normal due to proliferation of pigmented fibrous tissue and infiltrating plasma cells; the sclera was thickened to twice its normal dimension. The ciliary body showed partial regeneration. The iris was infiltrated by a few plasma cells and it was adherent in part to the lens where an anterior capsular cataract appeared to be forming. The cornea was normal.

The other eye of this hamster showed only a few cells in the anterior chamber and slight disorganization of the pigment in the ciliary body stroma.

Following lens extrusion, the left eye of hamster 386 presented a thick coat of sclera, choroid, and iris with much pigment, enclosing a cavity occupied by two large cushions of regenerating ciliary body epithelium and a small amount of fibrous scar tissue with calcific foci, pigment, and cholesterol clefts. Proliferating corneal epithelium had filled in the corneal ulcer and the pupil, pushing Descemet's membrane to the side.

Fig. 10 (Frenkel). Iridocyclitis with necrosis of adjacent retina, 29 days after infection with a noncyst-producing line of Besnoitia jellisoni (proliferative forms are present only). Note chorioretinal adhesions. (Hamster J-53-63 sulfadiazine treatment for 21 days. ×44. Periodic acid-Schiff followed by hematoxylin.)

Ocular lesions associated with Besnoitia infection

Clinical findings

Uveitis was of common occurrence *early* in the course of Besnoitia infection with noncyst-producing strains. As early as two weeks after infection and even while on sulfonamide therapy pigmented cells appeared in the anterior chamber and the clear black fundus reflex became first hazy and then white. This sequence took place rapidly, within less than a week.

With cyst-producing strains the first appearance of cells in the anterior chamber may occur later during infection and proceed more slowly; retinal vessels may remain visible for over a week especially when the retina has been detached. Eventually, the lens became opaque gray, and later a white or yellowish cataract was formed. Although both eyes frequently become involved at the same time, the lesions may appear independently of each other and each may progress at a different rate.

Incidence

Hamsters infected with noncyst-producing strains of Besnoitia showed greater than 50-percent eye involvement within a month. After two to three months hardly an eye remained univolved. With cyst-producing strains the rate of appearance appeared to be only slightly slower. In one experiment 18 out of 26 eyes of 13 hamsters showed signs of inflammation after two months.

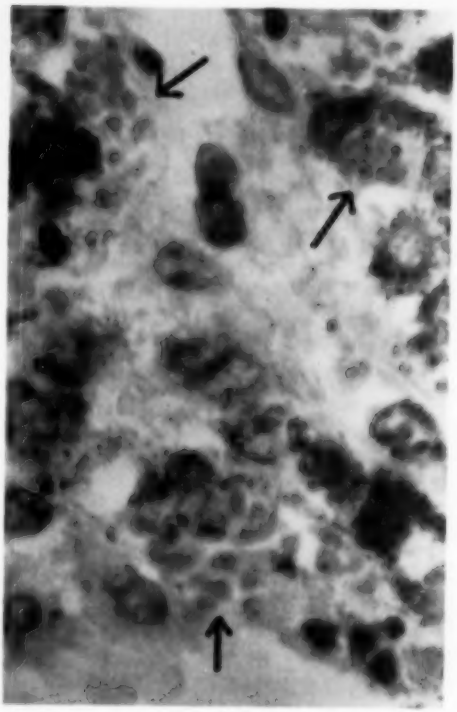


Fig. 11 (Frenkel). Cyclitis with proliferative forms of Besnoitia in the epithelial layer. Hamster J-54-5, 36 days after infection, 15 days after cessation of sulfadiazine therapy. (×1200. Hematoxylin-eosin.)

Fig. 12 (Frenkel). Cyclitis (A) with proliferative forms of Besnoitia spreading along the vessels in the ganglionic layer of the retina (B) and in the exudate (C) between external limiting membrane and retinal pigment epithelium. Hamster J-54-54, 20 days after infection, on sulfadiazine for entire period. (×130. Hematoxylin-eosin.)

LESIONS PRODUCED BY PROLIFERATIVE FORMS

By using strains of Besnoitia which had lost the power of producing cysts, the development of such lesions could be studied. There were nine eyes with an essentially normal retina accompanied by iridocyclitis (fig. 10).

In some of these eyes Besnoitia were numerous in the epithelium of the ciliary body (fig. 11). The stroma showed lymphocytic infiltration and disorganization of pigment. The iris contained a few lymphocytes. There were cells in the anterior and posterior chambers occasionally containing organisms. In four eyes, 17 to 37 days after infection, the ciliary body was partially necrotic and heavily inflamed with minimal retinal changes except in the adjacent pars plana.

From the ciliary body, Besnoitia, accompanied by inflammatory exudate, spread posteriorly along two routes. First they extended along the vessels in the ganglionic layer and on the retinal surface (fig. 12) and, secondly, between the external limiting and Bruch's membrane (figs. 12 and 13).

The second type of spread was associated with a sometimes massive cellular exudate composed of what appeared to be neutrophil granulocytes and macrophages, some of which contained organisms. This exudate dissected along Bruch's membrane, generally leaving it intact, but detaching eventually the entire retina (fig. 13).

Starting with the outer nuclear layer, the retinal lamination became more and more disrupted and organisms with inflammatory cells and epithelial pigment granules could be found progressively in all layers (fig. 14). Finally the retina became completely necrotic.

The choroid at the same time became progressively more infiltrated with lympho-

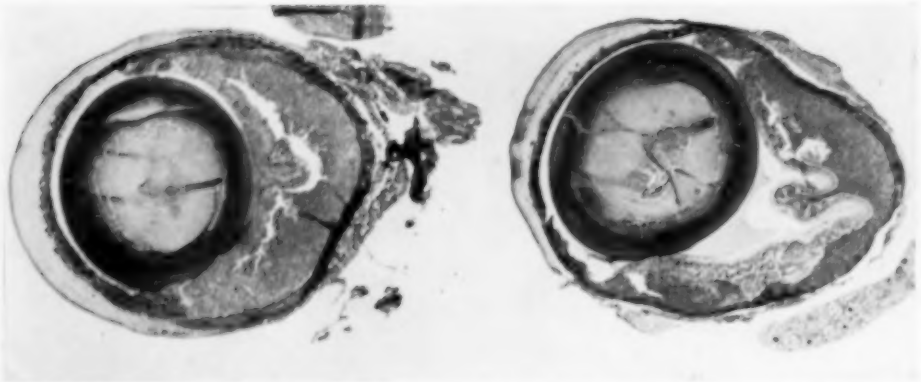


Fig. 13 (Frenkel). Further dissection of the retina by exudate; the retina is fairly well preserved in the eye on the right, but almost destroyed on the left by proliferating Besnoitia (see Figure 14). Iridocyclitis is far advanced. Hamster J-54-7, 37 days after infection, 16 days after cessation of sulfadiazine treatment. (×15. Hematoxylin-eosin.)

cytes and later with plasma cells, and possibly more pigment developed. Bruch's membrane frequently was found destroyed focally later during the necrotizing inflammatory process; however, neither organisms nor necrosis was ever seen in the choroid proper.

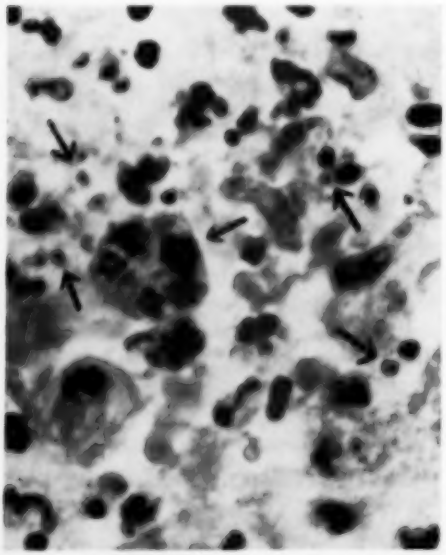


Fig. 14 (Frenkel). Proliferative forms of Besnoitia from central retinal remnant of eye on left of Figure 13. Arrows point to several of the free organisms and to nucleus of a cell containing four groups. (×1200. Hematoxylin-eosin.)

The sclera remained essentially uninvolved. The exudate in the anterior chamber increased, and so did the degree of iritis. Fibrin deposits were frequently found on the lens, together with cells and fibroblasts developing into posterior synechias. The lens began to degenerate posterior to its attachment to the ciliary body with the formation of "balloon cells" and later wrinkling and breaking of the capsule.

The entire eye was shrinking in size; the vitreous space frequently became obliterated with organization of the necrotic retina, joining the choroid to the posterior lens capsule. The ciliary body epithelium generally showed regeneration again covering the pigmented stroma (fig. 15); however, since fibroblasts were also attaching to the regenerated epithelial surface, its true epithelial nature was in doubt.

Lesions produced after infection with cyst-forming strains

Sections of 18 eyes from 10 hamsters were available five to 11 weeks after subcutaneous infection. Five of the animals had been autopsied and cysts were identified in the viscera and sometimes in the eye. Out of eight enucleated eyes, two were semiserially sectioned. Two cysts were found in one eye and in both serially sectioned eyes small

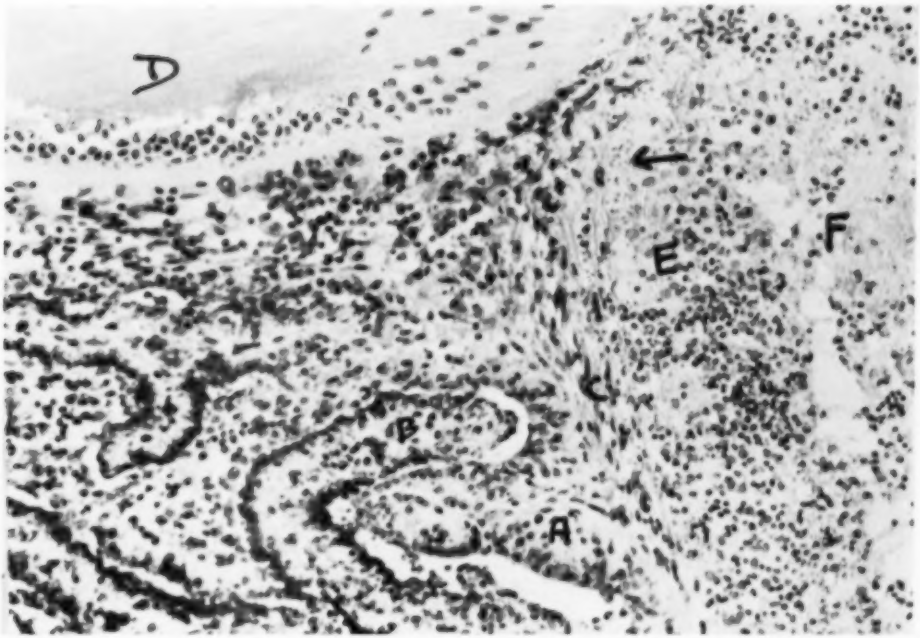


Fig. 15 (Frenkel). Regeneration of cells forming an epithelial-like covering (A) of the inflamed ciliary body stroma (B). Fibroblasts (C) attach to this "epithelium" and join it to the lens (D) and a retinal remnant (E). Proliferating Besnoitia are present in the retina adjacent to the lens (arrow). Necrotic debris and hemorrhage are shown in the vitreous space (F). Hamster J-54-13, 96 days after infection; sulfadiazine treatment during first 21 days. (×230. Hematoxylin-eosin.)

numbers of proliferative organisms were identified. While the lesions appeared some of the animals were on high doses of sulfadiazine which causes some inhibition of organismal multiplication.

The lesions in these eyes differed from those produced by proliferative Besnoitia:

First, the necrotizing lesions were focal in nature, and, second, cyclitis was rare. The latter lesion was observed in only two eyes and, in both, the ciliary-body and iris necrosis was part of a necrotizing endophthalmitis.

The focal lesions, however, characteristic of early eye involvement, were always near the posterior pole of the eye. The center of the lesion was formed by an area of retinal necrosis accompanied by subretinal edema and cellular exudate consisting of neutrophil granulocytes, lymphocytes, and macrophages (figs. 16 and 17). In only two of these foci were proliferative organisms found.

An inflammatory vesicle was often formed in the retina involving the pigment epithelium and Bruch's membrane, focally destroying both. Besnoitia cysts appeared to be located in the pigment epithelium layer (figs. 18 and 19). All cysts seen were intact. In some instances the choriocapillaris was necrotic. Away from the center of the vesicle part of the retina was preserved.

Some of the exudate dissected between Bruch's and the external limiting membrane for a short distance, forming chorioretinal adhesions. The choroid was infiltrated by neutrophil granulocytes, lymphocytes, and plasma cells, in the neighborhood of the retinal lesion. The sclera showed slight inflammation.

The parts of the retina uninvolved by necrosis, easily separated from the choroid

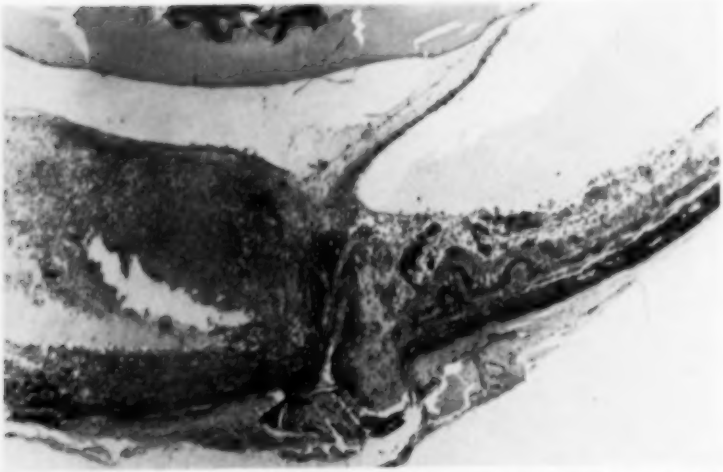


Fig. 16 (Frenkel). Large focus of necrotizing retinitis on one side of the optic nervehead. Onset of the lesion was between six and seven weeks after infection with a cyst-producing line; however no cysts were found in the eye and small numbers of proliferating Besnoitia accompanied this lesion. Hamster J-54-64 was treated for two weeks after infection, untreated for 25 days, and again treated with sulfadiazine for 12 days prior to enucleation. (×44. Periodic acid-Schiff followed by hematoxylin.)

during the process of fixation and dehydration. Here cellular infiltration was moderately severe to marked, especially perivascularly. Some of these cells resembled microglia more than hematogenous cells. The anterior segment was involved only by contiguity and a few cells were usually present in the anterior chamber.

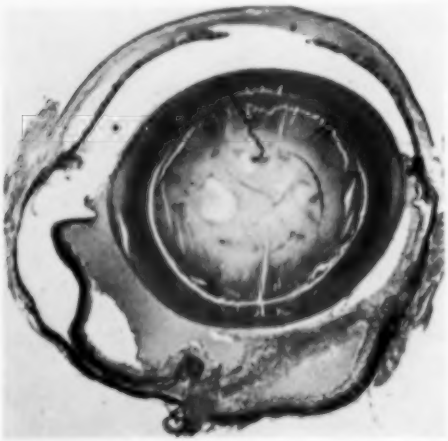


Fig. 17 (Frenkel). Chorioretinitis with inflammatory vesicle forming mainly on one side of the optic nervehead. The anterior segment is barely involved. Hamster J-54-63, six weeks after infection with a cyst-producing line of Besnoitia and treated with sulfadiazine for the first two weeks. (×44. Hematoxylin-eosin.) Figures 18 and 19, showing cysts, are from the same eye.

Repair of the necrotic retina was by fibrosis when arising from the choroid, and by gliosis if retinal tissue was remaining (fig. 20). The epithelial-like lining of the ciliary body regenerated, likewise. Detached retina that had become adherent to the lens showed degeneration of the outer nuclear layer. The lens showed degenerative changes and invasion by macrophages.

Besnoitia cysts persisted in the fibrotic retinal scar tissue (figs. 21 and 22). In one instance proliferative organisms were found to parasitize the lens epithelium (fig. 23); they were also present in several macrophages within the lens, but not elsewhere.

Discussion

A pattern of pathogenesis suggests itself despite differences in design of some of the experiments from which infected eyes were studied.

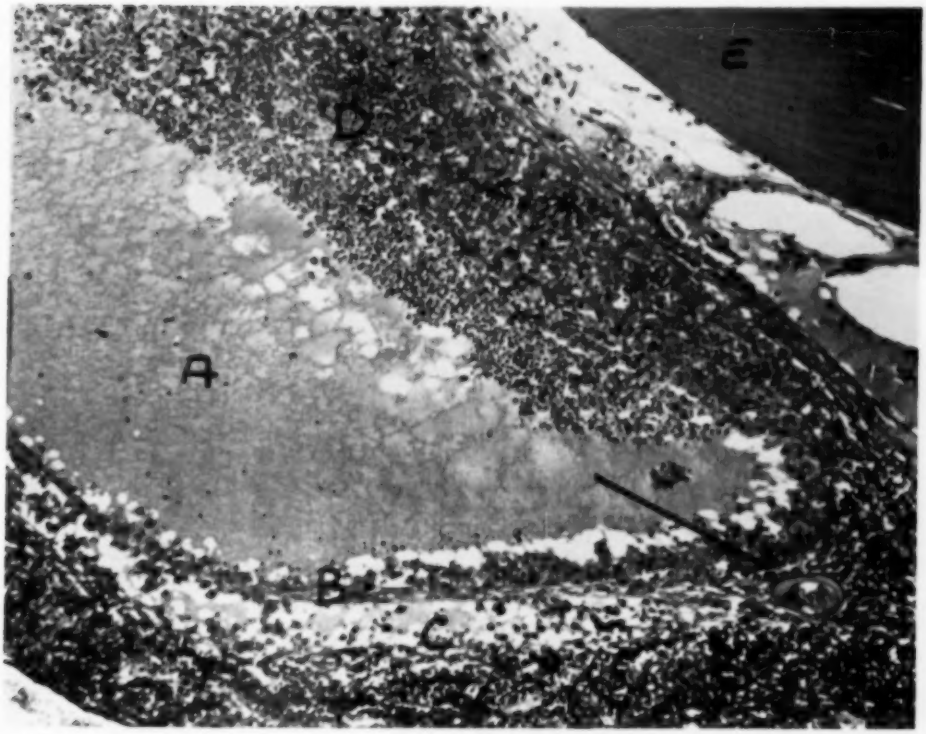


Fig. 18 (Frenkel). Close-up of chorioretinitis as shown in Figure 17 with retinal inflammatory vesicle (A) and young Besnoitia cyst (arrow.) The cyst appears to lie in the pigment epithelial layer, since some of the fibrils originating from Bruch's membrane (B) pass beneath it. The choroid (C) is inflamed. In the necrotic part of the retina (D) a few proliferative Besnoitia are present. (E) Lens. (Hamster J-54-63. ×130. Hematoxylin-eosin.) Figure 19 is also from this eye.

It is well to recall at the outset that in the hamsters studied both infections gave rise to ocular lesions at a time when lesions in the extraneural viscera were regressing. This was especially true of toxoplasmosis where eye lesions made their appearance, sometimes together with signs of encephalitis, months after initial infection. The latter had been treated with sulfadiazine until immunity was adequate to prevent death from extensive visceral lesions.

During Besnoitia infection eye lesions appeared earlier, sometimes when visceral lesions were at their peak. But it was clear that invasion of the eye was not a regular feature of acute infection, that it lagged behind the parasitization of other organs, and, most important, that organisms continued to proliferate and give rise to lesions in the eye long after they did in the other viscera, excepting the adrenal gland.

Toxoplasmosis

Early in toxoplasmosis eye lesions, when they occurred, were predominantly necrotic in character destroying large sections of the retina. Proliferative organisms were found. One such lesion was observed three months after infection; the other was seen two months after challenge with the RH strain, which is of different pathogenetic potential than the CJ strain of the initial infection.[6]

After the infection has persisted for several months, lesions can be produced by

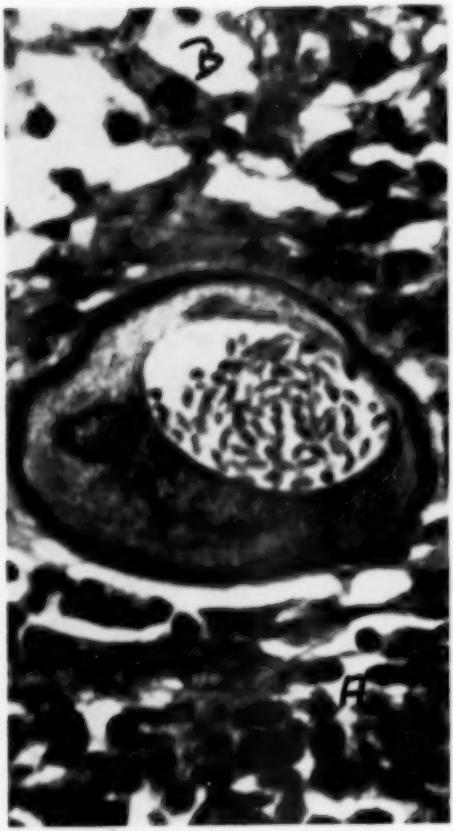


Fig. 19 (Frenkel). Another young Besnoitia cyst apparently situated in the retinal pigment layer. The heavy cyst wall is well shown, also the giant vesicular nuclei and the vacuole containing the crescentic organisms. Both choroid (A) and retina (B) are inflamed. (Hamster J-54-63. ×960. Hematoxylin-eosin.) Figures 17 and 18 are also from this eye.

proliferative forms as well as by cysts. Although both forms of the organism might be present, lesions appeared to be caused by only one or the other.

Several eyes showed the presence of intact Toxoplasma cysts, and, although no ruptured cysts were found, such are postulated to account for the lesions.

The similarity to the brain lesions in the same animals is suggestive; the apparent origin of the lesions in the retina where cysts are present, the chronic nature of the retinal lesions both as regards degenerative changes and the absence of acute inflammatory cells, and their widespread distribution suggesting multiple foci—these all favor the interpretation that an occasional ruptured cyst gave rise to these lesions.

The number of cysts in the retina is limited, and the intact cyst wall prevents chemotaxis. Only the sporadic release of antigen incident to cyst rupture can explain such necrotizing lesions followed by vesicle formation and gliosis in an eye in which cysts were present but proliferative organisms could not be demonstrated.

In the eye in which proliferative Toxoplasma were observed 10.5 months after infection, no cysts were found on semiserial sectioning. The lesion was single, focal, and acute, with organisms present only in or around the lesion. Many individual groups of parasites were seen. The destruction of individual host cells, their breakdown products, the liberation of antigen, and of organisms entering new cells, all participate in the production of this lesion.

It is difficult to decide with certainty whether this focal lesion was initiated by proliferating Toxoplasma reaching the retina via the blood stream, or by cyst rupture with the liberated organisms taking up proliferation. Only incomplete retinal necrosis was observed; nonetheless, the possibility exists that complete retinal necrosis, such as would be expected from the insult of cyst rupture, was missed in the 70-micron gap between sections.

The presence of chorioretinal adhesions however, rather than of separation by edema fluid, attests to a slowly progressing lesion, such as might be anticipated to result from slowly proliferating organisms and not from the sudden and massive antigen release due to cyst rupture.

The fact that proliferative organisms were not identified in lesions, and hence were probably absent when lesions occurred together with cysts, might indicate the in-

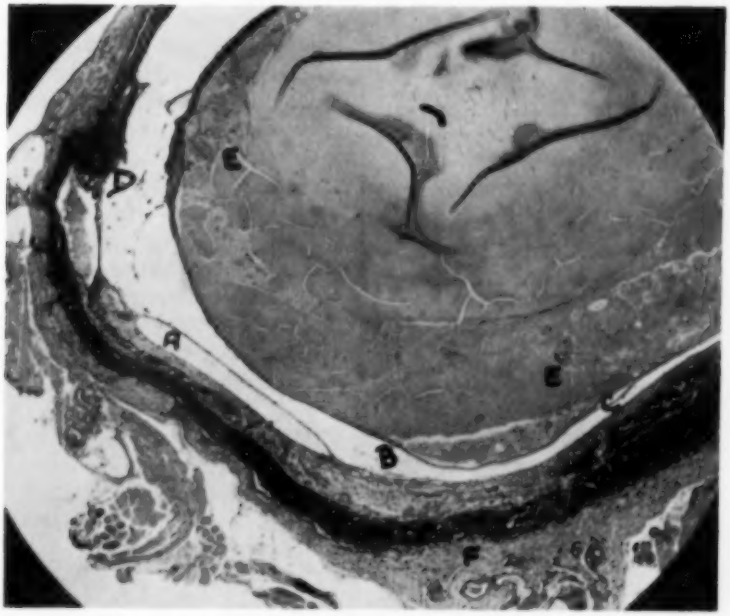


Fig. 20 (Frenkel). Repair of lesions by gliosis (B) adjacent to retinal remnant (A), and by fibrosis (C) next to area showing marked choroiditis. Next to the ciliary body (D) is an area of subretinal hemorrhage. Note degeneration of the lens (E), clinically evident as a grayish-white cataract. The vascular sclerosis (F) in the choroid is an effect of a desoxycorticosterone implant present for nine weeks. Duration of infection: 11 weeks. (Hamster P-129. DOCA-3. ×44. Periodic acid-Schiff followed by hematoxylin.)

ability of Toxoplasma to proliferate in that host. When proliferation does occur, these proliferation-inhibiting factors are apparently inoperative.

It makes a useful concept, consistent with presently known facts, to explain the early proliferation in the eye as due to lack of local immunity, the later chronic chorioretinitis associated with cysts that rupture as due to an essentially chemical inflammation in an immune-hypersensitive host, and the late focal lesions associated with proliferative forms as due to a reduced immune state in the eye.

It has previously been discussed[5,9] that the immune processes in the neuro-ectodermal derivatives of brain and eye lag behind those in the extraneural viscera. Hence, it is not surprising to find in hamsters that brain and eye lesions are still active when lesions in lung, liver, and spleen, for example, have regressed and healed two to three months after infection.

Attention has been drawn[9,10] to the persistence of cysts in the eye as an important feature in the pathogenesis of toxoplasmosis. The antigen released from cyst rupture appears analogous to the intradermal antigen injection in an immune-hypersensitive host. The late appearance in the eye of toxoplasmic proliferation is paralleled in hamsters by a similar process frequently taking place in the adrenal gland. A correlation of these two lesions in the same animal has not yet been made.

In chorioretinitis of human adults accompanied by the Toxoplasma-like parasites discovered by Mrs. Wilder, both cysts and proliferative forms have been described.[11] Since Toxoplasma has actually been isolated

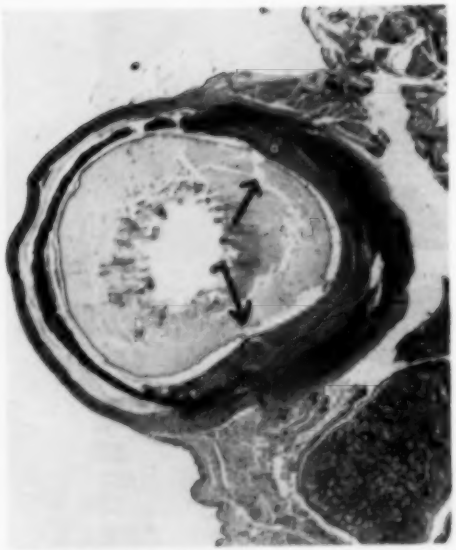


Fig. 21 (Frenkel). Advanced chorioretinal changes and repair by fibrosis, with two older Besnoitia cysts (arrows). Hamster 54-20, 11 weeks after infection. (×15. Periodic acid-Schiff followed by hematoxylin.) Figures 22 and 23 are from the same eye.

from one such eye,[12] there can be little doubt of the previous morphologic identification in some 50 cases,[11] which were characterized as a group also by the presence of antibodies to Toxoplasma measured by the dye test in all of the patients tested.[13]

The lesions described by Mrs. Wilder from the eyes of man[11] correspond in general to the lesions observed during the chronic infection of the hamster, especially those associated with proliferative forms. The relative role played by cysts and proliferative forms in toxoplasmic chorioretinitis of human adults has not yet been defined.

Besnoitia infection

Eye lesions due to Besnoitia in the hamster occur generally within the first three months of infection. In infections with proliferative Besnoitia, the lesion typically starts as cyclitis and spreads hematogenously and by exudation along Bruch's membrane to the posterior segment. Why the anterior segment is first involved here, whereas in toxoplasmosis lesions appear to originate posteriorly, has not been elucidated. Both organisms have in common the production of ultimately complete necrosis of the retina.

The pathogenesis of the focal necrotizing posterior pole retinitis associated with Besnoitia cysts has not yet been clarified. It might be initiated by the rupture of a cyst, just as has been discussed for Toxoplasma cysts whose apparent rupture leads to the formation of an inflammatory vesicle.

Lesions due to Besnoitia cyst rupture are commonly found in the viscera of many animals, since the thick cyst wall often persists until a granuloma is formed (fig. 23).

The relative paucity of cysts in the eye and the unavailability, so far, of many complete serial sections probably accounts for their not having been identified.* In addition, the relatively early appearance of these lesions would be compatible only with the rupture of small cysts, shortly after their formation, which would make it difficult to identify their remnants.

Ocular lesions and resultant ophthalmoscopic opacities incurred earlier during infection (figs. 17 and 18) preclude ophthalmoscopic detection of lesions resulting from persisting well-developed cysts (figs. 21 and 22) which might rupture later during the chronic phase of the infection.

Proliferative Besnoitia were found in the lesions discussed and may have persisted through the entire period of infection. However, their small number, the relative limitation of the lesion to the posterior pole, the formation of an inflammatory vesicle, as

* Recently, cyst rupture has been observed in the retina of a hamster. It occurred six and one-half months after infection. The large cyst which bulged above the retina had been under observation for over three months. It was found to be obscured by vitreous exudate four days after a satisfactory visualization. Histologic examination of the enucleated eye confirmed the clinical diagnosis. Lesions containing partially degenerated cysts, or merely remnants of cyst wall have also been observed since this paper was submitted for publication. These findings will be reported in detail.

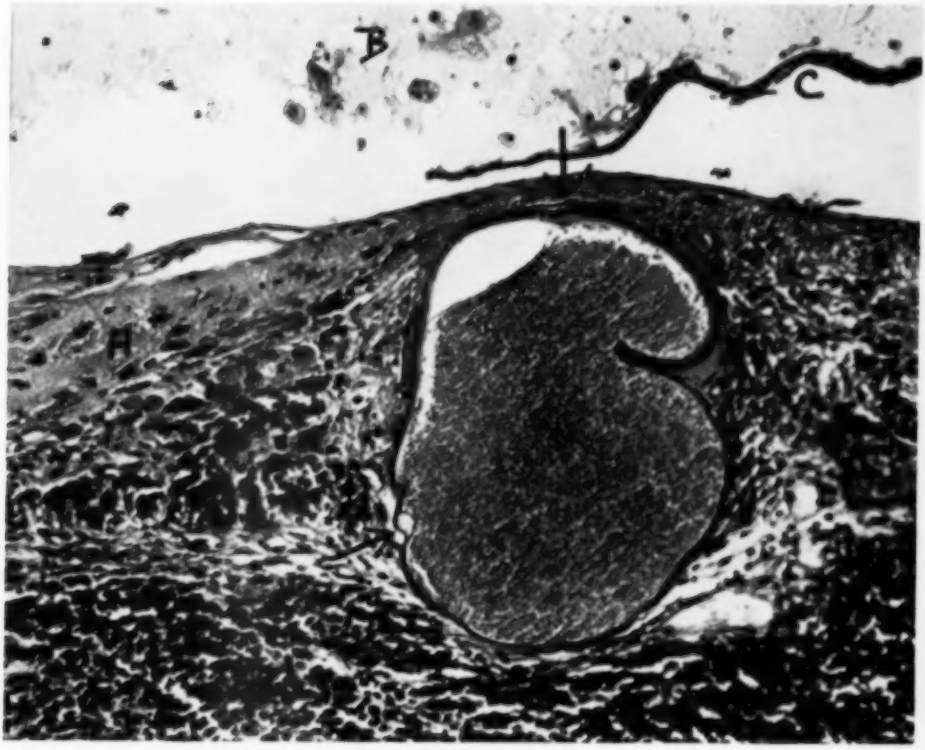


Fig. 22 (Frenkel). Old Besnoitia cyst almost filled with crescentic organisms and with two giant vesicular nuclei applied to inside of cyst wall (arrows). Fibrous replacement of retina (A), dispersal of pigment, degeneration of lens (B), and part of posterior lens capsule (C) are also shown. (Hamster 54-20. ×230. Periodic acid-Schiff followed by hematoxylin.) Figures 21 and 23 are from the same eye.

well as the absence of hematogenous spread or of cyclitis, so typical of infection with proliferative forms, all these make their primary pathogenetic participation unlikely.

When cysts rupture in extraneural tissues, the parasites leak out, stain poorly, and become necrotic, being phagocytized, and apparently do not enter new cells to resume proliferation. In the eye different conditions may obtain, preventing complete neutralization of liberated organisms and allowing for some proliferative activity.

In summary, cysts and proliferating Besnoitia have been found in chorioretinitic lesions during late chronic infection, just as in toxoplasmosis, and adrenal lesions due to proliferative Besnoitia have been observed likewise. However, Besnoitia is not known to occur in man. In cattle from which both infections have been described neither retinitis nor blindness have been recorded.

Host and tissue susceptibility

The peculiar susceptibility of the golden hamster and the production of chorioretinitis during the subacute and chronic infection with these two organisms, appears notable. Although this has not been investigated in detail such lesions have not been observed in most other animals under study. Only in pigeons has uveitis been observed with subacute Toxoplasma infection.[5] Paradoxically in that host, choroid, sclera, and extraocular muscles were involved by infection with the

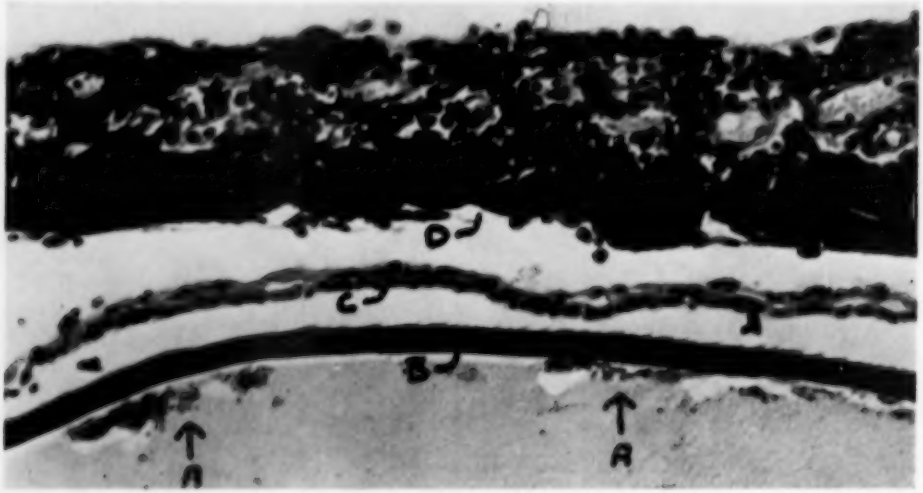


Fig. 23 (Frenkel). Proliferative Besnoitia parasitizing lens epithelium (A), which is probably the only ectodermal tissue remaining in this eye. They were also present in several macrophages from the posterior pole of the lens, similar to those shown on Figure 22. (B) Lens capsule. (C) Anterior capsular cataract. (D) Iris. Hamster 54-20, 11 weeks after infection. (×320. Periodic acid-Schiff followed by hematoxylin.) Figures 21 and 22 are from the same eye.

RH strain, whereas the retina was not. The absence of blood vessels from the retina of pigeons may explain that circumstance.

Tissue sensitivity has previously been discussed in relation to the peculiar prolonged susceptibility of neuroectodermal over other tissues. This is thought to be due in part to the blood-brain barrier restricting the perfusion of plasma protein containing antibody, and in part due to the less active cellular defense mechanism in the brain and eye.

More specifically, it is the neuro-ectodermal retina and the ciliary body epithelium which are parasitized by either organism, whereas the meningeal equivalents, choroid, ciliary body, supporting stroma, and iris, show inflammatory involvement by contiguity only.

Lymphocytes and plasma cells are the predominant cells present; only in the case of presumed cyst rupture with intense focal necrosis are what appeared to be neutrophilic granulocytes seen in the iris and choroid, parts of which are necrotic in these instances. Micro-organisms are also found in cells within the anterior and posterior chambers and the vitreous.

The parasitization by proliferative Besnoitia of the lens epithelium, after it has become accessible following rupture of the lens capsule, presents an interesting phenomenon. This was observed in an eye containing Besnoitia cysts, 11 weeks after infection. All neuro-ectodermal structures had been destroyed in that eye, with organization occurring from the mesodermal derivatives of the choroid.

Since the lens epithelium and the macrophages within the lens were the only cells parasitized, one wonders whether these were the only cells that remained susceptible. Their separation from antibodies due to the blood-brain (eye) barrier could account for the local status of immunity.

Repair is by gliosis if originating from the retina, and by fibrosis when extending from the choroid. Notable is the apparent process of regeneration of an epithelial-like covering of the pigmented ciliary body stroma. Since

these cells tend to form an epithelial-like pattern and contain pigment in some areas they may be thought of as neuro-epithelial melanophores.

Comparison of hamster and human toxoplasmosis

The usefulness of these two models of infection in the hamster depends on the demonstration that proliferative organisms as well as cysts of each organism may occur in the retina during the course of a generalized subacute and chronic infection. Furthermore, pathogenesis of the lesions appears to differ according to what growth form or forms of each of the organisms are present.

These models of infection therefore broaden our concepts of the possible pathogenesis of chorioretinitis in man which we know mainly from the histologic studies of eyes extensively involved, whether from adults or newborn babies. It brings into clearer focus the possibility that we may encounter clinically patients with chorioretinitis due to recurrent cyst rupture as well as due to a smouldering infection with proliferative forms of Toxoplasma.

Since no drug is at present available that will eradicate Toxoplasma cysts, we can only aim to minimize the tissue damage following cyst rupture whenever it occurs. Desensitizing immunization prophylactically and glucocorticoids therapeutically appear as the logical approaches, the rationale for which has been discussed.[8,10] Proliferating Toxoplasma are suppressed by appropriate chemotherapy, with synergistic combinations of sulfadiazine and Daraprim (a 2:4-diamino pyrimidine manufactured by Burroughs Wellcome & Co.) appearing as most promising.[14]

The evaluation of these modes of treatment in the animal models described is a problem for further investigation. Eye lesions in chronic toxoplasmosis of hamsters were not met frequently enough in recent experiments, although another strain of Toxoplasma, adaptation to the eye, or, less likely, a different line of hamsters might yield a suitable model for immunizing, hormonal, and specific chemotherapeutic studies.

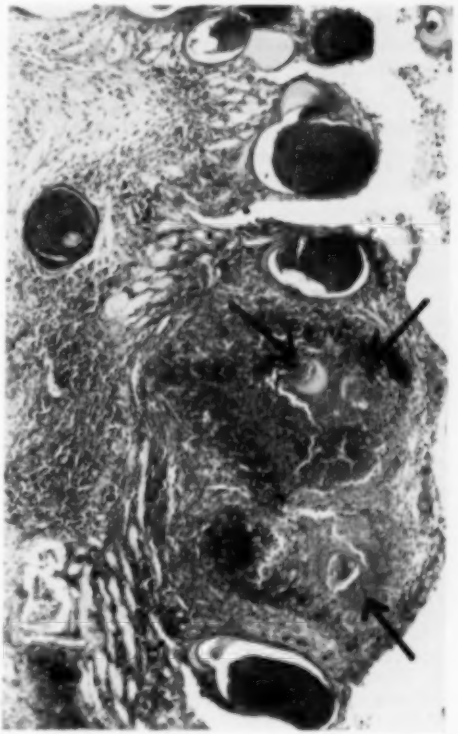


Fig. 24 (Frenkel). Granulomatous inflammation resulting from rupture of three cysts (arrows) in spermatic cord of mouse. Intact cysts are unaccompanied by inflammatory reaction. Mouse 50-282, two months after intraperitoneal inoculation (×130. Periodic acid-Schiff followed by hematoxylin.)

Intraocular inoculation of immune hamsters or rabbits may provide a more reproducible model to study treatment of infection with proliferative organisms. Very little is known about the immunologic aspects of chronic infection in hamsters as compared with man. Their natural immunity and defense mobilization potential is low and mortality is close to 100 percent with a majority of strains of Toxoplasma. However, degrees of acquired immunity appear to be similar to that in man, since chronic infections with most strains are inapparent.

In man we meet infection with apparent frequency but rarely clinical disease, hence the latter should be regarded as an unusual exception from the average host-parasite relationship, barring unusually pathogenic strains or heavy inocula. Hence symptomatic chronic infection of hamsters with the unusually pathogenic RH strain might parallel to some extent symptomatic chronic infection of man with unknown strains.

Antibody titers in hamsters are higher than they are in human adults with ocular lesions; Figures 5 to 8 are from animals with dye test titers of 1:65,000. Human infants, six to seven months after infection, might, however, have antibody titers in this range of magnitude. No correlation is available between antibody titers and activity of eye lesions in either man or animal.

Comparative endocrine relationships in man and animals need further study.

Although Besnoitia infection of the eye is quite reproducible in hamsters, there is no evidence that it occurs in man in any form. Nonetheless, being more reproducible and offering many parallels, this model may be of use in the study of treatment.

Summary

The appearance of ocular lesions in hamsters with chronic Toxoplasma infection by the RH strain as well as with subacute and chronic Besnoitia jellisoni infections are described. Proliferative forms and cysts occur in each of these protozoan organisms, and different lesions are present in association with each. The pathogenesis of each lesion is discussed, and similarities with the pathogenesis of toxoplasmic eye lesions in man are pointed out.

University of Kansas,
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Discussion

Helenor Campbell Foerster: This contribution by Dr. Frenkel is extremely interesting. Whenever an organism is shown to have the potentiality of causing eye disease in animals, we must think of the possibility of it causing similar intraocular inflammation in the human eye. Besnoitia is something to watch out for in the future.

As far as the individual protozoan is concerned,

I should like very much to see the Besnoitia organism, and compare it in size with that of Toxoplasma. Dr. Frenkel has told me that it is crescentic, as is the Toxoplasma organism, that it is only very slightly larger, and that it markedly resembles Toxoplasma.

I do not believe the cysts of Besnoitia could in any way be confused with those of Toxoplasma. They are much larger and should be readily apparent, I believe, on microscopic examination.

DR. LEON JACOBS: Dr. Frenkel's interesting paper indicates that as a model of recurrent toxoplasmosis or toxoplasmic chorioretinitis in humans, the eye infection in the hamster may serve better than the eye infection in other animals. In rabbits infected systemically by any peripheral route, we find much more difficulty in getting ocular lesions. Dr. Hogan reported some lesions in rabbits following intracarotid inoculation, but there was a very short period of time in which he had to operate because the animals generally succumbed within about 10 days.

We have tried intracarotid inoculation with an avirulent strain of Toxoplasma and have not been able to produce these eye lesions in the rabbit.

The occurrence of focal and inflammatory responses to the rupture of pseudocysts is probably the mechanism for the chronic recurrent bouts of inflammation seen in the human. On the basis of this rationale, the use of cortisone to control the disastrous chemotactic response, together with a chemotherapeutic agent to prevent proliferation of the parasites and to cause their destruction, should be explored.

The description of the pathogenetic events offered by Dr. Frenkel also serves to explain the difficulty any investigator would encounter in attempting to correlate ocular symptoms with a past systemic infection, which is what Dr. Sabin, who commented on my paper on Wednesday, would have us do.

Ocular lesions generally do not occur until the immune response to the infection has dropped to a relatively low level. Antibody production apparently takes place in tissues physiologically remote from the central nervous system, so that, after the infection may be burned out in the extraneural viscera, encysted parasites may persist in the eye and brain and eventually may rupture out of the pseudocysts and again proliferate.

On the basis of this thesis it may be worth while to explore the usefulness of intramuscular injection of dead antigens to restimulate systemic antibody production.

As a model for the chemotherapeutic work aimed at finding satisfactory treatment for ocular toxoplasmosis, however, these studies on the hamster present certain difficulties. First, there are uncertainties in the production of lesions. Second, there is necessity for treatment with sulfonamides in order to arrive at the chronic state, which means that the parasites with which you want to deal chemotherapeutically have already been exposed to sublethal concentrations of the drug. Third, there is difficulty in clinical evaluation of the lesions on any, even a semiquantitative, basis.

In our laboratory we are approaching this problem of chemotherapy with the use of a relatively avirulent strain inoculated into the anterior chamber of the rabbit eye. While the RH strain so inoculated will rapidly kill the animals, this strain allows survival, and we are finding that the appearance of lesions and the course of the inflammatory response over a period of four to six weeks is generally predictable.

Of corollary interest from the standpoint of immunity in the ocular tissues, we have found that anterior chamber challenge with the RH strain (the virulent strain) after previous immunization with the other strain, results in the same severe anterior uveitis. In contrast to this, there is hardly any local reaction following challenge by peripheral routes.

DR. J. K. FRENKEL (closing): I would like to thank both Mrs. Foerster and Dr. Jacobs for their discussion. I omitted saying, and am glad Mrs. Foerster pointed it out, that the proliferative forms of Toxoplasma and of Besnoitia are probably indistinguishable on sections; one can make a distinction perhaps on smears. The cysts are easily differentiated, since those of Besnoitia are larger, have a thicker cyst wall, and are lined by cyst-wall nuclei.

Secondly, it should be mentioned that these organisms give rise to different serologic responses. One can differentiate infections with the dye test. An immunologic test, such as a skin test, may be useful in certain animals and man if the infection is a chronic one.

I also agree with Dr. Jacob's remark, that for chemotherapeutic studies the hamster model is not as useful as one where infection can be quantitatively introduced into the eye. The model I have described has its principal value in the study of pathogenesis, and in pointing out what can happen during the natural course of an infection.

I believe the approach that Dr. Jacobs is using is more direct and less complicated, and should bring forth results of interest to those who are concerned with treatment.

STUDIES OF IMMUNITY IN EXPERIMENTAL HERPETIC KERATITIS IN RABBITS*

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Herpes-simplex virus infection of the cornea in man characteristically produces a superficial branching (dendritic) ulcer, corneal hypesthesia, and a mild conjunctivitis. The benign clinical course of this lesion is thought to depend upon circulating humoral antibody, and in recurrent ulcers, upon fixed tissue antibody in the cornea. Occasionally in adults, and frequently in children, herpes simplex corneae occurs as a primary lesion in an individual lacking humoral antibody. In these cases the keratitis and secondary conjunctivitis are usually severe, resembling the primary keratitis seen in rabbits following corneal inoculation with the virus.

The mechanism of immunity in herpes-simplex infections of the cornea is not clear. Braley[1] believes that humoral immunity is relatively unimportant in corneal herpes, and that local tissue immunity plays an important part in recovery from infection and in prevention of recurrences.

There are several interesting reports of experiments on corneal immunity using foreign proteins. Thompson[2] and co-workers injected egg albumin into rabbit cornea and demonstrated that precipitins could be formed in the corneal stroma. Thompson and Olson[3] injected hen ovalbumin in to the right cornea of a rabbit and human serum albumin into the left. The respective corneal tissues contained only antibodies against the antigens with which each had been injected.

Bursuk[4] injected typhoid bacilli into rabbit cornea and found that agglutinins and opsonins appeared in the corneal tissues earlier, and in greater concentration, than in the serum.

Moro[5] studied corneal immunity in vaccinial keratitis by means of keratoplasty. In his experiments he grafted corneas from normal to immune animals and attempted to reinfect the grafted eyes. There was no evidence of infection following reinoculation. He also grafted corneas from immune to normal animals and found that the corneas could be infected as readily as the nongrafted eyes. He concluded that the immunity resulting from vaccinial keratitis was systemic in type rather than of the cornea alone.

Loewenstein[6,7] conducted experiments on corneal immunity in herpetic keratitis in the rabbit. In his experiments the previously infected eye when healed was immune to further inoculation with herpes-simplex virus. The interval between inoculations was not stated.

Doerr and Vochting[8] inoculated the second cornea of eight rabbits with herpes-simplex virus at intervals between 15 and 30 days after primary inoculation of the first cornea. In these animals the reaction in the second cornea tended to be less severe and the incubation period slightly prolonged. These workers did not record any observations of the comparative residual scarring in the two eyes.

Florman and Trader[9] reported that a high level of humoral antibody appears in rabbits within three weeks of corneal inoculation with herpes-simplex virus. The level of antibody determined in their experiments reached its peak in six weeks and no drop was noted within nine weeks.

Since humoral antibody and local tissue immunity are considered to be important factors in preventing recurrence and in recovery from herpetic infections in the human cornea, a further study of this problem, using rabbit cornea, has been undertaken.

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Methods and Materials

The strain of herpes-simplex virus used in these experiments was originally isolated from a dendritic ulcer of a five-year-old girl. This strain was chosen because it did not produce encephalitis in rabbits, making it possible to carry out the experiments without animal deaths from this cause.

All rabbits were inoculated by scarifying the cornea with a No. 18 hypodermic needle and placing a small piece of cornea from the 11th rabbit passage of the virus on the scarified area.

Procedure and Results

I. Serologic studies

In order to determine the degree of humoral immunity resulting from a primary herpetic keratitis, 14 animals were bled before inoculation and at monthly intervals thereafter. Serologic studies were carried out by the neutralization technique described by Ruchman.[10] The Kärber method of calculating the LD_{50} was used.[11,12]

From Chart 1 we see that serum antibody in the rabbits had reached a high level by one month after primary inoculation, reinoculation did not increase the antibody, and the level was maintained for at least two months after the second inoculation. These values were comparable to those reported by Florman and Trader.

II. Reinoculation experiment

To study the effect of immunity resulting from a primary herpetic keratitis in rabbits upon reinoculation of the same cornea, 66 previously infected eyes were reinoculated at intervals of from three to 24 weeks.

As a control, the effect of trauma was assessed by scarification of the corneas of 14 animals at similar intervals after the primary herpetic keratitis had subsided. All corneas were examined daily with the slit-lamp.

From Chart 2 we see that it was possible to reinfect 42 percent of the eyes with the virus. The majority of these reinfections were manifested by a mild keratitis, in marked contrast to the severe primary keratitis previously observed. No significant reaction was seen in any of the scarified control eyes.

III. Corneal inoculation of animals with humoral immunity

In order to determine the effect of humoral antibody on a normal cornea in the

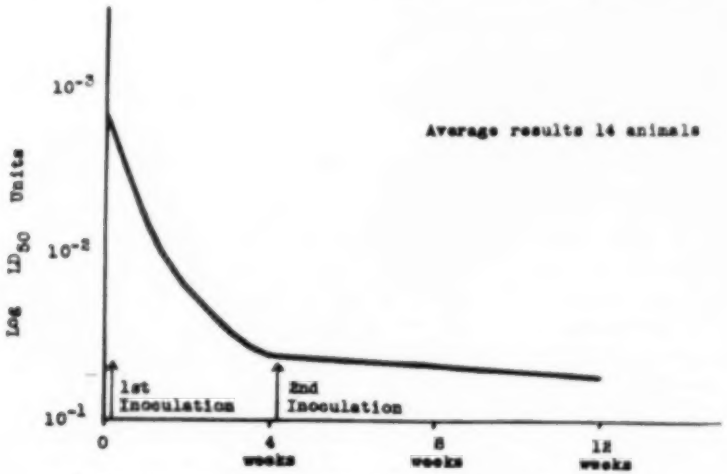

Chart 1 (Hall, MacKneson, and Ormsby). Serum neutrilization tests.

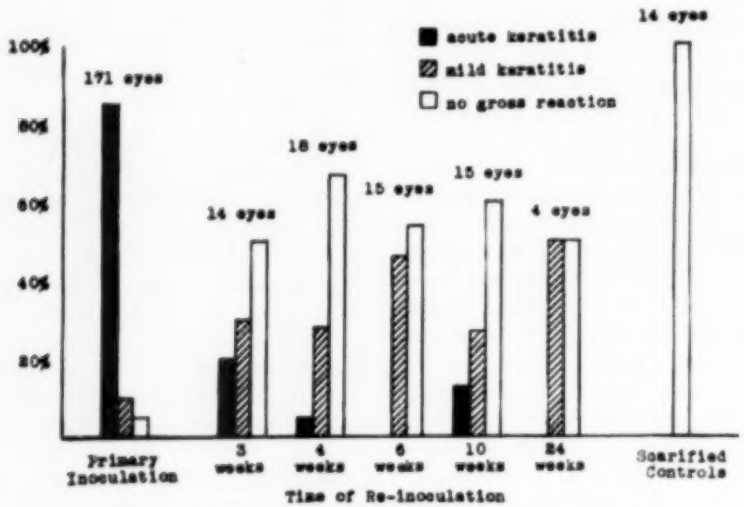

Chart 2 (Hall, MacKneson, and Ormsby). Reaction of reinoculated eyes.

immune animal, 22 rabbits, which had recovered from a primary herpetic ketatitis in the right eye, were inoculated in the left eye after intervals of four, eight, and 22 weeks.

From Chart 3 we see that a typical primary type of keratitis developed in almost all the eyes despite the presence of a high level of humoral antibody. However, the degree of residual corneal scarring in these eyes was much less than that resulting from primary herpetic keratitis.

IV. Studies of immune corneal grafts in nonimmune animals

In an attempt to determine the effect of tissue immunity in the absence of humoral antibodies, full thickness corneal grafts were taken from 20 eyes which had recovered from a primary herpetic keratitis, and transplanted into the corneas of nonimmune rabbits. Eight nonvascularized grafts were reinoculated four weeks after transplant.

Gross observation, slitlamp microscopy, and histologic study of these grafted corneas failed to show any significant difference in the degree of keratitis between the graft and the host cornea. It will be seen from Chart 4, that seven of the eight eyes developed keratitis.

V. Studies of nonimmune corneal grafts in immune animals

To study the effect of combined humoral and adjacent tissue immunity on transplanted rabbit cornea, 20 full-thickness corneal grafts were taken from nonimmune eyes and transplanted into healed corneas which had recovered from a primary herpetic keratitis. Four weeks after transplant 10 nonvascularized grafts were inoculated with herpes-simplex virus. Following gross and slitlamp observation of these grafted corneas, the eyes were enucleated for histologic study on the fourth day.

In Chart 5 it will be seen that the inflammatory reaction was minimal in both the graft and the host cornea. This was similar to the previously observed effect of reinoculation in healed eyes.

Discussion

From these experiments it is clear that circulating antibody, in the rabbit, plays a small but important part in reducing the severity of herpetic keratitis. This is demon-

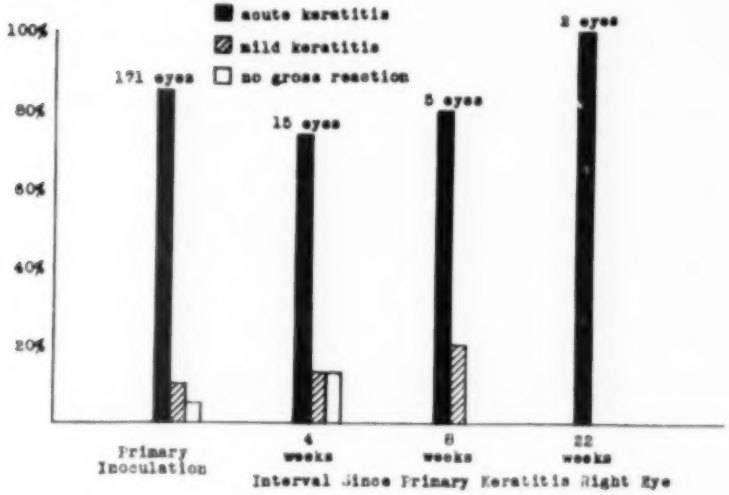

Chart 3 (Hall, MacKneson, and Ormsby). Reaction of inoculated left eyes.

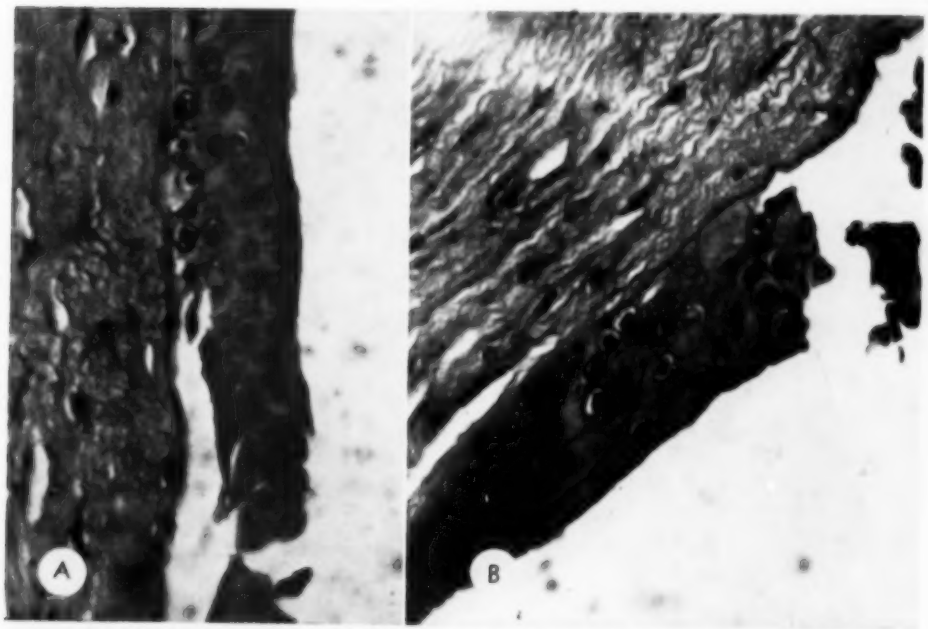

Fig. 1 (Hall, MacKneson, and Ormsby). (A) Host cornea (nonimmune). (B) Corneal graft (immune). These two microphotographs show the presence of herpetic inclusion bodies in the epithelial cells of both graft and host cornea.

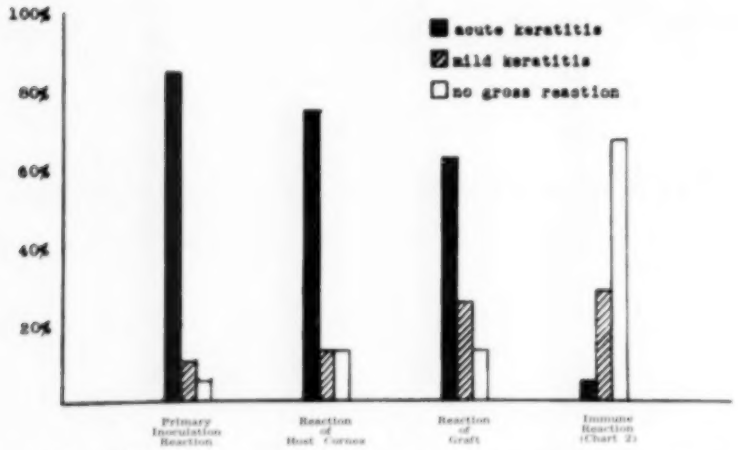

Chart 4 (Hall, MacKneson, and Ormsby). Inoculation of grafts immune to nonimmune cornea.

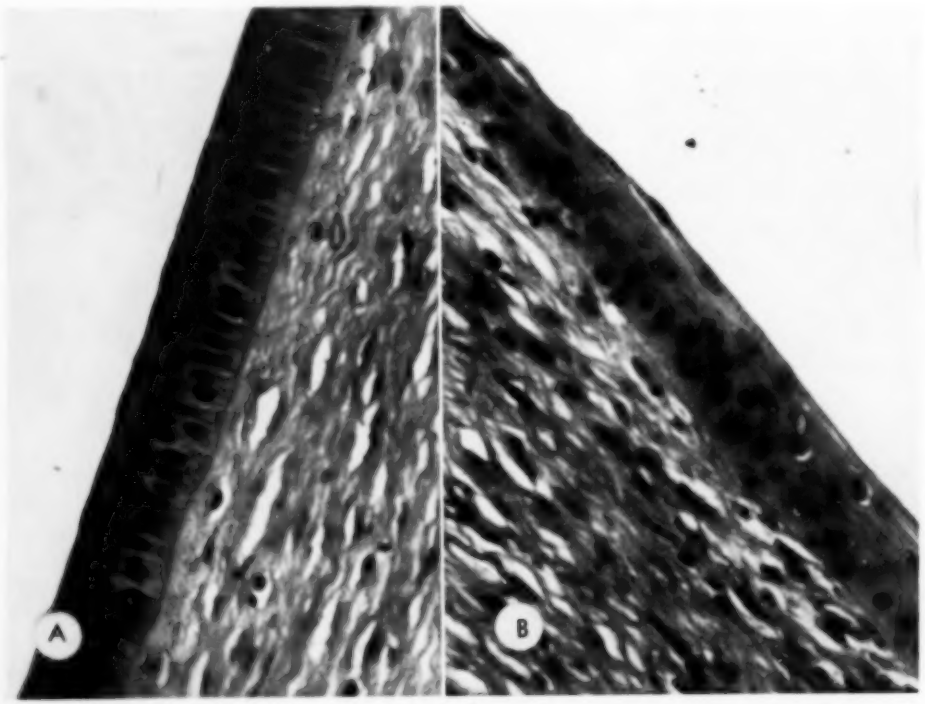

Fig. 2 (Hall, MacKneson, and Ormsby). (A) Host cornea (immune). (B) Corneal graft (nonimmune). These microphotographs show the absence of typical herpetic inclusion bodies in the epithelial cells of both graft and host cornea.

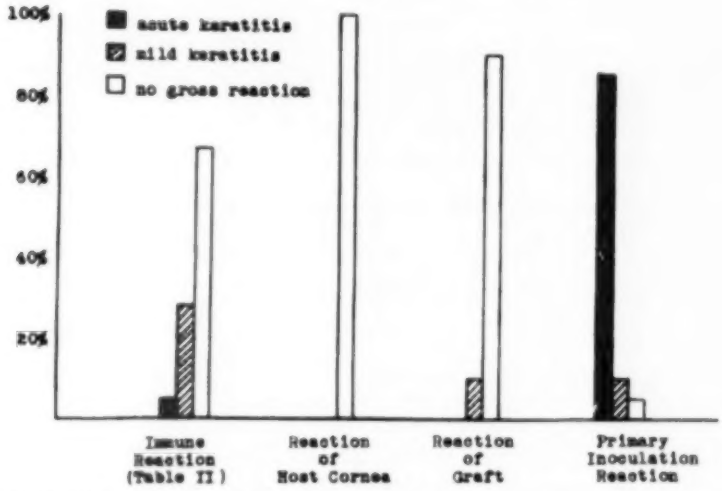

Chart 5 (Hall, MacKneson, and Ormsby). Inoculation of grafts nonimmune to immune cornea.

strated by the reduction in residual scarring which occurs when the second eye of a rabbit is inoculated with the virus after the first eye has recovered from a primary herpetic keratitis.

Local defense mechanisms apparently play the major role in reducing the severity of herpetic keratitis. This is clearly demonstrated upon reinoculation of the eyes which had recovered from a primary keratitis. Whether this tissue immunity is due to fixed antibodies or to the presence of latent virus in the cornea, the so-called interference phenomenon, is not clear at this time.

The failure of immune cornea to retain its immunity when transplanted into a nonimmune host and the ability of a nonimmune cornea to acquire immunity when it is transplanted into an immune eye is of practical importance in keratoplasty, particularly in regard to the transplantation of human cornea with herpetic scarring. The explanation for this observation is not clear, but it seems possible that there may be a replacement of the cells in the donor cornea by those of the host.

Summary

1. Primary herpetic keratitis in the rabbit resulted in a high serum antibody titre by the fourth week following inoculation. Reinfection did not produce any increase in humoral antibody. The high antibody level persisted for at least three months.

2. After recovery from a primary keratitis, reinoculation of the same cornea resulted in reinfection in 42 percent of the corneas. The majority of these infections were of a mild type. The remaining 58 percent of eyes did not show evidence of reinfection.

3. Inoculation of the cornea of the second eyes which were protected by humoral immunity alone resulted in a keratitis typical of the primary reaction, but the residual scarring of these corneas was less marked than in the first eyes which had recovered from a primary keratitis.

4. Previously inoculated corneas transplanted into nonimmune animals did not retain their immunity to the virus upon reinoculation. Nonimmune corneas transplanted into previously inoculated eyes acquired the immunity of the recipient eye.

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The authors wish to express their appreciation to Mrs. Grace Walter for technical assistance in the preparation of histologic material.

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Discussion

Dr. Alson E. Braley: To me, this is a most fascinating study, since for quite a while I was of the opinion that the local antibody in the cornea was extremely important as far as reinfection is concerned.

While it is not directly analogous that rabbits and human beings can be compared as far as herpes infection is concerned, I believe this study will lend a great deal of support to some of the theories that we have expounded for some little time.

We still do not understand the entire mechanism of immunity in herpes, and the role played by the circulating antibodies certainly has been minimized in the human being in comparison to the rabbit. As has been pointed out here today, in the rabbit the circulating antibody seems to play a rather important role, since reinfection of the second cornea was not as severe as the first infection.

Although reinfection of the second cornea in the same rabbit did occur with fair regularity, reinfection of the first cornea after healing did not occur with as great regularity, therefore indicating the presence of local immune bodies.

I had planned to report some of the work that perhaps some of you might not be too well acquainted with, namely, that of Medawar, a man who did work on a strain of inbred rats, in which he attempted to transplant rat skin from one area to another.

As all of you know, homologous tissue when transplanted (particularly skin) will die. Transplantation of identical twins or autogenous tissue, of course, lives very readily. One can develop a strain of rats that is so completely inbred that transplanting of skin from one rat to another rat will take place without any death. Then, by taking an outside series that is not inbred, it is impossible to transplant those rats.

Where is all this getting us? It is getting us to the source of differentiation between local antibodies and general antibodies and circulating antibodies. From this work it was indicated that the source of the local antibody was not from the local tissue itself, but was probably from the lymph node.

I have wondered how the local antibodies could get from the lymph nodes into the cornea. As far as I know, there is no way in which the cornea can develop antibodies except from the local histiocytes, if such is possible.

Herpes simplex, as you all know, is probably one of the most important diseases, and the immunity to herpes in the human being should be studied considerably. One of the methods of studying this, of course, is with corneal transplants. However, I spoke to Dr. Maumenee this morning, and from his work and from other indications it would appear that the host cornea contributed to the graft and therefore might prevent the development of infections in the cornea.

In both instances, whether it was an immune cornea that was transplanted into a normal rabbit or a normal rabbit cornea transplanted into an immune animal, the new cornea would then take on the characteristics of the animal with which it was then living.

This has been a most interesting paper, and in my opinion it contributes to the theory and knowledge of our work with herpes simplex.

DR. JAMES H. ALLEN: I would like to congratulate the authors on a beautiful piece of work, which I hope is the beginning that will clear the confusion about the immunity reactions to herpes infections of the cornea.

Actually, from the theoretical standpoint the immunologist could have predicted these results from the clinical reactions we see. However, for the first time, I believe, we have definite, experimental proof of the value, though limited, of circulating antibody but I do not see that there is any proof in the paper that the prolonged local resistance was due to a local antibody effect.

From the clinical literature one gains the impression that the transplantation of corneal tissue into an eye which has been infected with herpes simplex is not likely to be followed by a herpetic infection of the transplant. Two years ago I had the unfortunate experience of having herpes-simplex infections develop in the grafts of two patients in whom I had performed corneal transplantation procedures. The infections developed between four and 10 days following the operation. In reviewing the history of the donor patients, there was no indication of herpes infections, therefore the virus must have been present in the recipient's eyes.

I think it is important to correct the impression in the clinical literature that herpes-simplex infection does not occur in grafted corneas.

DR. HENRY F. ALLEN: I agree with Dr. Braley and Dr. Allen in commending the authors for a very stimulating piece of work, which bears on the basic question of the distinction between tissue antibody and humoral antibody.

Because of the fundamental nature of this work, it seems particularly important to strive for an exact parallelism in controls. I wonder whether the authors have transplanted two immune corneas from the same animal into a rabbit which had been infected on one cornea, and whether they have done the same with two nonimmune corneas. The implications of such an experiment might be complementary to their previous observations.

Following the demonstration in 1948 by Heil, Allen, and Cheever of the presence of herpes neutralizing antibody in gamma globulin, we tried the therapeutic effect of injecting gamma globulin into human patients with dendritic keratitis. Our results were disappointing.

We feel that in a primary dendritic keratitis, or in the later sequence of developments in a cornea infected with herpes, systemic injections of gamma globulin have no therapeutic value. A possible exception exists in the case of a cornea heavily vascularized as a result of chronic or oft-repeated infections. The theoretic basis, of course, is that the added blood supply serves to carry the antibody to the virus. We have had a few encouraging results in such cases.

To determine whether administration of gamma globulin systematically would be effective on a prophylactic basis, as suggested by Dr. Stone, would require repeated injections over a long period of time. In view of the variability of incidence of attacks of herpes-simplex keratitis, which we know can appear at intervals of six and even more years, the results would be extremely difficult to evaluate.

DR. RAY L. HALL (closing): On behalf of Dr. Ormsby, Mrs. MacKneson, and myself I wish to thank the discussers for their very informative comments about this paper. Although the hour is late, I would like to clear up a misconception regarding the results of one of our experiments, which would seem apparent from the remarks of the discussers.

From our observations, we were not able to attach great importance to the role of circulating humoral antibody. The only significant effect we could see was the reduction in residual corneal scarring. The reaction of the cornea was in most instances very similar to that of a nonimmune eye.

Some statement which I made may have created a different impression but I think that the results are fairly clear in the written paper.

PENETRATION OF MAGNAMYCIN® INTO THE AQUEOUS HUMOR OF RABBITS*

HANS R. HAUSLER, M.D., AND HUGH L. ORMSBY, M.D.
(With the technical assistance of Ingrid Munck)
Toronto, Ontario

The new antibiotic Magnamycin® is a crystalline monobasic substance obtained from products of the mold Streptomyces halstedii. Active in inhibiting gram-positive bacteria it has little effect against gram-negative organisms. The growth of Rickettsia and the larger viruses is inhibited by Magnamycin, properties otherwise only shared by the broad-spectrum types of antibiotics.

Magnamycin does not have cross resistance to other antibiotics of general use and test organisms become resistant to it only in a slow stepwise fashion.[1] It is generally effective against those gram-positive bacteria found to be highly resistant to other antibiotics.[2,3] In experimental animals the toxicity of Magnamycin has proved to be low.[3,4]

Clinically, it is an effective therapeutic agent in the treatment of infections caused by gram-positive organisms,[5] and has particularly proved its therapeutic value in the treatment of enterococcic urinary tract infections.[6,7]

The promising results obtained by Halliday and Ormsby[8] in the treatment of infectious external eye diseases by topical administration of Magnamycin ophthalmic ointment made a more detailed study of the antibiotic as applied to ophthalmologic problems desirable. The purpose of this investigation was to study the penetration of Magnamycin into the aqueous following administration by various methods.

In five groups of experiments, aqueous concentrations of the antibiotic were determined after topical administration of two different ophthalmic ointments, after intramuscular and intravenous injection, after subconjunctival injection, and after administration by iontophoresis and by corneal bath. Finally a number of experiments were undertaken to study the effect of Magnamycin in the treatment of intraocular infections. In all experiments rabbits were used as test animals.

METHODS AND MATERIALS

The Magnamycin preparations used in the experiments were supplied by Chas. Pfizer & Co., Brooklyn, New York. The following preparations were used:

1. Magnamycin Hydrochloride Ophthalmic Ointment, containing 10 mg. Magnamycin hydrochloride per gm.

2. Magnamycin Ophthalmic Ointment, containing 10 mg. Magnamycin as the base per gm.

3. Magnamycin Intravenous, 10 ml. vials, containing 200 mg. Magnamycin as the hydrochloride.

4. Magnamycin—Brand of Carbomycin, for intramuscular use, 10 ml. vials, containing 500 mg. Magnamycin as the sulfate.

5. Diagnostic Buffered Magnamycin, 20 ml. vials, containing 20 mg. Magnamycin as the phosphate for standardization purposes.

In some of the experiments dealing with the subconjunctival injection of the antibiotic or with its administration by iontophoresis, hyaluronidase† was added to the Magnamycin solutions in the manner speci-

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* From the Departments of Ophthalmology and Bacteriology, University of Toronto.

† The hyaluronidase used was Wydase (lyophilized hyaluronidase, manufactured by J. Wyeth & Brother Limited, Walkerville, Ontario, supplied in ampules containing 150 TR (turbidity reducing units) each.

fied below. Heparin* served as an anticoagulant in the assay of plasma, 10 international units being added to each ml. of rabbit blood. The animals were anesthetized by intravenous or intraperitoneal injection of Nembutal.† In addition, or as an alternative, ether for anesthesia was used on occasion.

For the removal of the aqueous, 0.25-ml. glass syringes with 27-gauge needles were found suitable.

The subconjunctival injections were carried out by using a 27-gauge needle on a 0.50-ml. glass syringe, the total volume of fluid injected being 0.20 or 0.25 ml. respectively.

Iontophoresis was carried out with a lucite eyecup electrode similar to the one described by Selinger.[12]

The Magnamycin in the aqueous and plasma was assayed following a modification of the twofold serial dilution method described by Rammelkamp,[9] the medium being Hartley broth. The inoculum per tube was 0.05 ml. of a standard culture of Staphylococcus aureus (Peoria), usually diluted 1,000 or 10,000 times in the Hartley broth. The incubation time was 24 hours at 37°C. At the end of this period the tubes were inspected for the inhibition of gross turbidity.

In the experiments where Magnamycin was used in the treatment of experimental endophthalmitis, von Sallmann's[10] method was followed to produce a standard intraocular infection. The infecting organism was Staphylococcus aureus (Peoria).

1. Magnamycin—topical administration

As previously reported by Halliday and Ormsby,[8] the repeated administration of ophthalmic ointment containing Magnamycin as the hydrochloride salt into the conjunctival sac of rabbits produced concentration levels in the aqueous ranging from 1.25 to 5.0 μg. per ml. These experiments were repeated on five animals (10 eyes) and similar results were obtained.

Approximately 0.2 gm. of the ointment was administered eight times successively into the lower conjunctival sac with an interval of 15 or 20 minutes between treatments.

As can be seen from Table 1, concentrations up to 5.0 μg. Magnamycin per ml. of aqueous were obtained. The average aqueous concentration in six eyes after eight treatments at 15-minute intervals was 1.66 μg. per ml., and in four eyes at 30-minute intervals 2.81 μg. per ml.

Magnamycin ophthalmic ointment containing the antibiotic as the base was tested on 24 animals (48 eyes). The experimental conditions and results obtained are also presented in Table 1. In these tests no measurable aqueous concentrations were obtained, even after intensive treatment. Moderate conjunctival injection and slight chemosis occurred regularly after treatment with either of the two Magnamycin ophthalmic ointments, and no appreciable difference was found in this respect.

2. Magnamycin—systemic administration

The aqueous and plasma of a total of 36 rabbits was assayed for Magnamycin after the animals had received the antibiotic by intramuscular and intravenous injection. The Magnamycin concentrations in the aqueous and plasma obtained under varying experimental conditions are presented in Table 2 and Table 3.

When Magnamycin was administered intramuscularly, and one single intramuscular injection was given, aqueous and blood were removed either 90 or 120 minutes after the injection. Ninety minutes after a dose of 25 mg. Magnamycin per kg. of animal, the average aqueous concentration in eight eyes was 0.41 μg. per ml. and after a dose of 50 mg. Magnamycin the average aqueous

* Produced by the Connaught Medical Research Laboratories, 1.0 ml. containing 1,000 international units of heparin.

† Pentobarbital sodium, Abbott Laboratories Limited.

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TABLE 1

AQUEOUS CONCENTRATIONS FOLLOWING TOPICAL ADMINISTRATION
(Magnamycin)

Experiment	No. of Eyes Tested	Type of Magnamycin Ointment	Number of Applications and Interval between Same		Time between Last Application and Removal of Aqueous	Aqueous	
			Do.	Interval	Minutes	Concentration Range (μg./cc.)	Concentration Average (μg./cc.)
1	8	Base	1		120		<0.62
2	6	Base	1		60		<0.62
3	4	Base	1		30		<1.25
4	10	Base	8	15′	45		<0.62
5	10	Base	8	15′	60		<0.31
6	6	Base	8	15′	60		<0.62
7	4	Base	8	30′	60		<0.62
6a	6	Hydrochloride	8	15′	60	1.25–2.50	1.66
7a	4	Hydrochloride	8	30′	60	1.25–5.00	2.81

concentration in four eyes was 0.62 μg per ml. Injection of 100 mg. per kg. body weight produced an average aqueous concentration of 0.83 μg. per ml. in six eyes after 90 minutes, and an average concentration of 0.93 in four eyes after 120 minutes. In one experiment two animals received 10 minutes of shortwave treatment to both eyes, this treatment being initiated 50 minutes after the injection of 100 mg. Magnamycin per kg. In these animals the average aqueous concentration was found to be 1.08 μg. 90 minutes after the intramuscular injection. In a similar experiment three animals received shortwave treatment of 15 minutes, also initiated 50 minutes after the injection and the aqueous was removed 120 minutes after the intramuscular injection. The average concentration of Magnamycin in these six eyes was 1.66 μg.

In brief, the Magnamycin concentration in the aqueous was about 40 percent of that of the plasma 90 or 120 minutes after a single intramuscular injection of the antibiotic.

In the experiments where the animals were subjected to shortwave treatment before the aqueous was removed, the Magnamycin in the aqueous was above 50 percent of the concentration found in the plasma. Under the experimental conditions involved, shortwave treatment increased the penetra-

TABLE 2

AQUEOUS CONCENTRATIONS FOLLOWING SYSTEMIC ADMINISTRATION
(Magnamycin as the sulfate—intramuscular injection)

Experiment	No. Animals	No. of Eyes	Dose (mg./kg.)	Time Interval between Injection and Removal of Aqueous (minutes)	Aqueous		Plasma	
					Concentration (μg./cc.)	Concentration (μg./cc.)	Concentration (μg./cc.)	Concentration (μg./cc.)
1	4	8	25	90	0.31–0.62	0.41	0.62–1.25	1.09
2	2	4	50	90	0.62	0.62	1.25	1.25
3	3	6	100	90	0.62–1.25	0.83	1.25–2.50	2.08
3a(SW1)	2	4	100	90	0.62–1.25	1.08	1.25–2.50	1.87
4	2	4	100	120	0.62–1.25	0.93	2.50	2.50
4a(SW2)	3	6	100	120	1.25–2.50	1.66	1.25–5.00	2.80

SW1—10 minutes shortwave treatment to both eyes, started 50 minutes after injection.
SW2—15 minutes shortwave treatment to both eyes, started 50 minutes after injection.

TABLE 3

AQUEOUS CONCENTRATION FOLLOWING SYSTEMIC ADMINISTRATION. MAGNAMYCIN AS THE HYDROCHLORIDE—INTRAVENOUS INJECTION

Experiment	No. Animals	No. of Eyes	Dose (mg./kg.)	Time Interval between Injection and Removal of Aqueous (minutes)	Aqueous		Plasma	
					Concentration Range (μg./cc.)	Concentration Average (μg./cc.)	Concentration Range (μg./cc.)	Concentration Average (μg./cc.)
1	3	6	25	45	0.62–2.50	1.37	2.50–5.0	3.5
2	2	4	25	45				
3	3	6	50	45	0.62–5.00	1.98	5.0–10.0	7.5
4	3	6	50					
5	2	4	100					
6	2	4	100	45	1.25–5.00	2.91	5.0–20.0	12.5
7	2	4	100					
8	3	6	200	45	2.50–5.00	3.54	10.0–20.0	16.6

tion of Magnamycin into the aqueous.

In all experiments where Magnamycin was administered intravenously, the aqueous was removed 45 minutes after the injection.

The average aqueous concentration in 10 eyes after an intravenous injection of 25 mg. Magnamycin per kg. body weight was 1.37 μg. per ml., and after a dose of 50 mg. per kg. body weight the average concentration in 12 eyes was 1.98 μg. per ml. When 100 mg. per kg were injected, the average aqueous concentration in 12 eyes was found to be 2.91 μg. per ml., and after an intravenous dose of 200 mg. Magnamycin the average aqueous concentration in six eyes was 3.54 μg. per ml.

Generally, when aqueous concentrations were expressed as a percentage of corresponding plasma concentrations, higher relative aqueous levels were found after intramuscular than after intravenous administration, under the experimental conditions involved.

3. MAGNAMYCIN—SUBCONJUNCTIVAL INJECTION OF THE HYDROCHLORIDE

In 52 animals (104 eyes) the Magnamycin concentration in the aqueous was determined after subconjunctival injection of the hydrochloride salt. Table 4 shows the results obtained under varying experimental conditions.

Subconjunctival injection of 50 mg. Magnamycin in a volume of 0.25 ml. saline produces in the rabbit severe irritation of the conjunctiva which is occasionally followed by local necrosis and marginal corneal opacity. At a dosage of 10 mg., 20 mg., or 25 mg., the inflammatory reaction is less pronounced. It is mild and transient at a dosage of five mg.

The addition of five to 10 TR units of hyaluronidase to the injection fluid reduced the inflammatory reaction and also led to increased bulbar penetration of the antibiotic. Comparative concentrations of Magnamycin in the aqueous were consistently higher in the 26 animals where hyaluronidase had been added to the injection fluid.

In the rabbit a single subconjunctival injection of five mg. Magnamycin hydrochloride with five TR units of hyaluronidase in 0.2 ml. of saline produced good therapeutic levels of the antibiotic in the aqueous, lasting up to four hours after injection.

4. MAGNAMYCIN—ADMINISTRATION BY IONTOPHORESIS

Levels of Magnamycin in the aqueous were determined on a total of 234 eyes, the

TABLE 4

AQUEOUS CONCENTRATIONS FOLLOWING SUBCONJUNCTIVAL INJECTIONS

(Magnamycin)

Experiment	No. Animals	No. of Eyes	Dose (mg.)	Hyaluronidase (Dose TRU)	Fluid Volume (saline inj. ml.)	Time Interval between Injection and Removal of Aqueous (minutes)	Aqueous	
							Concentration Range (μg./cc.)	Concentration Average (μg./cc.)
2	6	12	5		0.2	90	2.5 –10.0	5.6
8	2	4	5	5	0.2	60	5.0 –10.0	7.5
8a	2	4	5	5	0.2	120 (2 hr.)	5.0 –10.0	7.5
8b	2	4	5	5	0.2	240 (4 hr.)	2.5 – 5.0	4.3
9	2	4	5	5	0.2	120 (2 hr.)	5.0 –10.0	7.5
9a	2	4	5	5	0.2	240 (4 hr.)	2.5 – 5.0	3.7
9b	2	4	5	5	0.2	360 (6 hr.)	0.62– 1.25	0.93
9c	2	4	5	5	0.2	720 (12 hr.)	<0.31	<0.31
9d	2	4	5	5	0.2	1018 (18 hr.)	<0.31	<0.31
1	6	12	10		0.2	90	5.0 –20.0	11.0
7	2	4	10	5	0.25	90	10.0 –20.0	12.6
5	4	8	20		0.2	90	10.0 –20.0	10.5
7a	2	4	20	5	0.25	90	20.0 –40.0	25.0
3	5	10	25		0.2	90	10.0 –40.0	18.0
6	3	6	25	10	0.25	90	20.0 –40.0	26.6
4	5	10	50		0.25	90	20.0 –80.0	42.0
6a	3	6	50	10	0.25	90	20.0 –80.0	50.0

aqueous being removed five minutes after iontophoresis or corneal bath. Prior to the paracentesis the eyes were thoroughly washed with sterile saline. The Magnamycin applied during the iontophoresis was dissolved in saline, since in preliminary experiments Magnamycin dissolved in distilled water produced severe corneal opacity. It was likewise found in preliminary experiments that anode iontophoresis yielded higher aqueous concentrations than cathode iontophoresis.

Solutions containing the hydrochloride and the sulfate salt of the antibiotic were also investigated for their penetration properties. Three different concentrations (20 mg. per ml., 10 mg. per ml., and 5.0 mg. per ml.) were used with both the sulfate and the hydrochloride salt. Further variables were introduced by the use of four different amperages (1.5, 2.5, 5.0, and 10.0 milliamperes), the period of time during which the iontophoresis was applied (for five and 10 minutes), and in some experiments, the addition of hyaluronidase to the solutions.

The Magnamycin iontophoresis was very well tolerated. Even a solution containing 20 mg. of the antibiotic per ml. of saline, applied at 10 ma. for 10 minutes did not produce gross corneal opacity. The results obtained under all these variables are presented in Table 5.

Generally, the addition of hyaluronidase to the solutions leading to a concentration of five TR units per ml. increased the aqueous concentrations of Magnamycin only slightly.

On one occasion the Magnamycin concentration was found to be less after the enzyme had been added to the test solution.

A solution containing 10 mg. Magnamycin hydrochloride or sulfate, applied for five minutes at 2.5 ma. produced therapeutic levels well above those necessary to inhibit most strains of the gram-positive organisms.

No clear-cut difference was found in the

TABLE 5

AQUEOUS CONCENTRATIONS FOLLOWING IONTOPHORESIS AND CORNEAL BATH

	Magnamycin in Saline (mg./cc.)		No. Eyes	10 ma-10' (μg./cc.)	No. Eyes	5 ma-10' (μg./cc.)	No. Eyes	5 ma-5' (μg./cc.)	No. Eyes	2.5 ma-10' (μg./cc.)	No. Eyes	2.5 ma-5' (μg./cc.)	No. Eyes	1.5 ma-10' (μg./cc.)	No. Eyes	1.5 ma-5' (μg./cc.)
Magnamycin hydrochloride	20	i	6	33.30	—	—	—	—	—	—	—	—	—	—	—	—
		c	2	2.50	—	—	—	—	—	—	—	—	—	—	—	—
	10	i	6	21.60	6	12.5	4	7.50	6	7.50	4	4.37	6	3.33	6	2.50
		c	2	1.87	—	—	2	0.62	—	—	2	<0.62	—	—	—	—
	10 & Wy	i	4	25.00	—	—	—	—	—	—	6	4.57	4	4.37	—	—
		c	2	2.50	—	—	—	—	—	—	—	—	—	—	—	—
	5	i	6	15.00	6	8.33	4	5.00	6	4.58	4	2.14	6	2.50	4	1.60
		c	2	1.25	—	—	2	<0.62	—	—	2	<0.62	—	—	—	—
	5 & Wy	i	4	15.00	—	—	—	—	—	—	—	—	—	—	—	—
		c	2	1.25	—	—	—	—	—	—	—	—	—	—	—	—
Magnamycin sulfate	20	i	6	30.00	—	—	—	—	—	—	—	—	—	—	—	—
		c	2	2.50	—	—	—	—	—	—	—	—	—	—	—	—
	10	i	6	18.30	6	13.30	4	8.70	6	8.33	4	5.00	6	4.16	6	2.71
		c	2	1.87	—	—	2	0.62	—	—	2	<0.62	—	—	—	—
	10 & Wy	i	4	20.00	—	—	—	—	—	—	2	5.42	4	4.16	—	—
		c	2	1.25	—	—	—	—	—	—	—	—	—	—	—	—
	5	i	6	15.00	6	7.50	4	4.30	6	4.16	4	1.87	6	1.86	4	1.56
		c	2	1.25	—	—	2	<0.62	—	—	2	<0.62	—	—	—	—
	5 & Wy	i	4	12.50	—	—	—	—	—	—	—	—	—	—	—	—
		c	2	1.25	—	—	—	—	—	—	—	—	—	—	—	—

i—iontophoresis
c—corneal bath (control)
Ma.—Milliamperes
Time—' (minutes)
Wy—Wydase

penetration properties of the two Magnamycin salts.

Corresponding aqueous concentration levels obtained after corneal bath were generally less than 10 percent of those obtained after iontophoresis.

TREATMENT OF INTRAOCULAR INFECTIONS WITH MAGNAMYCIN

In a group of experiments intraocular staphylococcal infection was produced on a total of 48 rabbits with the technique referred to above. Of these animals, 39 received treatment with Magnamycin, administered by various routes, while nine were left untreated serving as controls. All control eyes were lost within a period of five days.

EXPERIMENTAL PROCEDURE

1. Topical administration. In five animals Magnamycin was applied topically. Treatment was started 30 minutes after the inoculation, and consisted of four successive administrations of ophthalmic ointment containing the antibiotic as the base. The ointment was applied into the lower conjunctival sac, the interval between treatments being 15 minutes.

2. Intramuscular administration. Treatment in five animals was started four hours after infection and consisted of two daily injections of 100 mg. Magnamycin as the sulfate per kg. body weight for two days.

3. Subconjunctival injection. In this experiment treatment consisted of two successive subconjunctival injections of 5.0 mg. Magnamycin hydrochloride and five TR units of hyaluronidase in a volume of 0.25 ml. saline. The interval between the two injections was six hours.

This treatment was initiated:

2 hours after infection in 3 animals
4 hours after infection in 3 animals
6 hours after infection in 4 animals
12 hours after infection in 5 animals
18 hours after infection in 5 animals

4. Administration by iontophoresis. Treatment with a solution containing 10 mg. Magnamycin as the hydrochloride per ml. of saline was started 30 minutes after infec-

TABLE 6

MAGNAMYCIN

TREATMENT OF EXPERIMENTAL INTRAOCULAR INFECTIONS

(Staphylococcus aureus Peoria)

Treatment Magnamycin		Number of Eyes Treated	Results Excellent	Intermediate	No Effect
Ophthalmic ointment		10	0	1	9
Intramuscular injection		10	0	6	4
Subconjunctival injection—					
First treatment after infection: (hours)					
2		6	4	1	1
4		6	3	2	1
6		8	3	3	2
12		10	0	6	4
18		10	0	2	8
Iontophoresis					
(Ma.)	(Min.)				
10	10	6	4	1	1
2.5	10	6	4	1	1
2.5	5	6	3	1	2

See text for experimental procedure and interpretation of results.

tion—on three animals a current of 10.0 ma. was applied for 10 minutes; on three animals a current of 2.5 ma. was applied for five minutes; on three animals a current of 2.5 ma. was applied for 10 minutes.

RESULTS

The results obtained are listed in Table 6 and were expressed under similar headings as those used by Locke.[11] Under the heading, "Excellent Results," are therefore listed those eyes, which by the fifth day after inoculation did not show signs of active intraocular inflammation. "Intermediate Results" were obtained on eyes responding to treatment to a lesser degree, and eyes listed under the heading "No Effect" had developed severe suppurative endophthalmitis by the fifth day after infection.

SUMMARY

The penetration of Magnamycin into the aqueous humor of rabbits was studied. Aqueous concentrations of the antibiotic were determined after topical administration of two different ophthalmic ointments, after intramuscular and intravenous injection, after subconjunctival injection, and after administration by iontophoresis or corneal bath.

When ophthalmic ointment containing Magnamycin as the hydrochloride was used, the antibiotic was found to penetrate into the aqueous, whereas no measurable penetration occurred after the use of ophthalmic ointment containing Magnamycin as the base.

When administered by the intramuscular route, the penetration of Magnamycin into the aqueous was found to be increased after the eyes had been subjected to shortwave treatment. When aqueous concentrations were expressed as a percentage of corresponding plasma concentrations, higher relative aqueous levels were found after intramuscular than after intravenous administration.

Subconjunctival injection of Magnamycin as the hydrochloride produced high aqueous concentrations, but caused considerable local irritation. The addition of hyaluronidase reduced the inflammatory reaction and also

led to increased bulbar penetration of the antibiotic.

Iontophoresis led to high concentrations of Magnamycin in the aqueous. It was well tolerated when saline solutions of Magnamycin were used, but Magnamycin dissolved in distilled water produced severe corneal opacities. Anode iontophoresis yielded higher aqueous concentrations than cathode iontophoresis.

In a number of experiments Magnamycin has proved effective in the treatment of staphylococcal intraocular infections.

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GROWTH OF CORNEA IN TISSUE CULTURE*

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Tissue culture methods have been used extensively as a substitute for living animals in the isolation and cultivation of viruses. In this study human corneal and conjunctival tissues have been grown in tissue culture and are to be used in an attempt to cultivate viruses, such as those of epidemic keratoconjunctivitis, superficial punctate keratitis, inclusion conjunctivitis, and trachoma. Thygeson (1939) reported the successful growth of human corneal and conjunctival epithelium and the subculture of fibroblasts.

The following report contains only the results of the work with cornea and is a description of the various changes that were made in the physical conditions and chemical composition of the medium in order to improve conditions for the growth and subculture of corneal epithelium and fibroblasts. Although monkey cornea was used for the basic study, most of the findings proved applicable to human cornea.

MATERIALS AND METHODS

Cornea. Human cornea was obtained chiefly from cadavers at the time of autopsy, but also from eyes enucleated in the operating room and from embryos. Monkey cornea was made available to us in large quantity from the Connaught Medical Research Laboratories, Toronto.

Tissue culture techniques. Two types of culture flasks were used: roller tubes and Carrel D 3.5 flasks. The roller-tube technique consisted of implanting the tissue in a thin clot of chicken plasma and chick-embryo extract covering the inside of a test tube. After a period of five to 10 min., 2.0 ml. of fluid medium was added and the tubes were rotated in a horizontal position. The Carrel D 3.5 flasks were used either with or without a plasma clot and with 1.0 to 2.0 ml. of fluid medium. All cultures were incubated at 37°C.

RESULTS

MONKEY CORNEA

1. *Physical conditions*

a. *Epithelium*

i. *Size of tissue implanted.* Various sizes and depths of corneal cuttings and discs were taken to determine the optimum method of preparing the tissue for the growth of epithelium. It was found that from cuttings of cornea that were chopped with scissors into small pieces, only very small epithelial sheets, measuring one to two mm. in diameter, could be grown. Moreover, only about 20 percent of the pieces produced epithelium. When an attempt was made to reduce the chopped cornea to single cells and cell-aggregates by digestion with the enzyme trypsin, it was found that instead of the pieces breaking up, they imbibed water, became very gelatinous in nature, and stuck one to another in a large viscous mass which could not be implanted.

Successful growth of corneal epithelium was obtained with large corneal cuttings collected by the method of Eastcott et al., 1954. The results showed that the larger the cutting, the better the growth. New growth of epithelium was first visible after 24 hours' incubation and reached a maximum size in three to four days if the outgrowth was poor, or seven to 10 days when growth was very good. From pieces having a diameter of eight mm., the epithelium extended between seven and 12 mm. outward from the edge of the tissue and surrounded the implant in a continuous sheet.

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Full-thickness or penetrating discs taken with a trephine did not grow as well as extremely thin cuttings taken with a scalpel or Graefe knife. Epithelium alone, with no supporting substantia propria, grew poorly. If the cuttings were implanted epithelial side down, there was no growth, and if the epithelium was damaged or scraped off, there was no growth from the denuded area. These are probably the reasons why chopped cornea grew poorly, for, during chopping, the epithelial layer may be scraped off some of the pieces and others may be implanted epithelial side down.

The preferred technique, therefore, is to use large thin corneal cuttings with an intact epithelial surface implanted up. This finding in regard to the size of the implant is in contrast to most other tissues which have been grown in tissue culture. The fact that cornea is a nonvascular tissue probably makes it possible to use such large pieces without damage by anoxia of the central cells.

ii. *Depth of plasma clot.* Various depths of plasma clot were tried. When corneal cuttings were implanted in a deep clot, there was little or no epithelial growth, as Thygeson (1939) had shown. The cells seemed to pile up around the edge of the tissue instead of spreading out in a sheet as they did when a very shallow clot was used. The shallow clot was to a large extent digested by the cells.

When the corneal cuttings were attached to the center of the flasks with small droplike plasma clots the same size as the cuttings, it was found that the outgrowth was usually a little more irregular than with the large shallow clot, and, on the average, growth was slightly less. However, the cells themselves were far clearer for microscopic examination and for staining. If a small drop of specially purified agar (Dulbecco and Vogt, 1954) was used instead of plasma to hold the tissue in place, there was a marked reduction in amount of growth. Therefore, the plasma clot was probably contributing essential nutrients as well as serving to hold the tissue in place.

The best technique for encouraging epithelial growth is to use a shallow clot covering the bottom of the Carrel flask or inside of the roller tube. However, if the cells themselves are to be examined critically or are to be stained, the small droplike clot should be used.

b. *Fibroblasts*

It was found that fibroblasts grew readily from the substantia propria of cornea whether the implanted tissue was chopped or in large cuttings, but they took a long time to appear. This time varied greatly and was related to the amount of epithelial growth. When epithelial growth was small, they appeared in three or four days, but when the epithelial growth was extensive, the fibroblasts often did not appear for seven to 10 days. The fibroblasts pushed the epithelial sheet out and formed a solid mass of cells close to the original implant. Then either the fibroblasts stopped growing and both cell types disintegrated, or the fibroblasts overgrew the epithelium, which later disintegrated, leaving a pure culture of fibroblasts.

c. *Subcultures of epithelium and fibroblasts*

It was found that pure cultures of the two kinds of cells could be obtained by the following method. A corneal cutting was cultured long enough to permit proliferation of the epithelium. Just before the fibroblasts appeared the original cutting was lifted out leaving a pure culture of epithelium surrounding the site of the cutting. The original cutting was transplanted into another flask, where, in three or four weeks, fibroblasts from it overgrew the entire bottom of the flask. To subculture these fibroblasts, the original cutting was first lifted out and then the layer of fibroblasts was scraped out with a spatula in what looked like stringy wisps. These could either be teased apart or cut into pieces and implanted in new flasks.

The original flask with the epithelium ceased to proliferate once the corneal cutting was removed. The epithelial sheet remained intact, however, for a period of up to a month (34 days being the longest time recorded) and then disintegrated, the cells pulling apart and finally rounding up. Attempts to subculture the epithelial sheets in the same way as the fibroblasts were not successful. Recently the enzyme trypsin has been used to break up the large sheets, and epithelial cells have now been successfully transplanted and maintained through three subsequent passages.

2. *Chemical composition of the medium*

a. *Synthetic media*

Synthetic medium No. 199 (Parker, 1950) was tried, but proved to be inadequate for the growth of both epithelial cells and fibroblasts. Synthetic medium No. 703 (Healey et al., 1954) has not as yet been tried. When the oxidation-reduction potential of medium No. 199 was adjusted by increasing the amount of glutathione, cysteine hydrochloride, and ascorbic acid, as in synthetic medium No. 703, no better growth resulted.

b. *Semisynthetic media*

Semisynthetic media were tested and it was found that by adding a small amount of horse serum (final concentration, two percent) and chick-embryo extract (final concentration, 0.5 percent) to synthetic medium No. 199, a good medium for growing and subculturing fibroblasts was obtained, but this medium was only fair for growing epithelium. Other modifications were also tried. Aqueous humor from cow's eyes was added to medium No. 199 in various amounts and it was found that 20-percent aqueous humor was equally as good as two-percent horse serum. When the chick-embryo extract was replaced with a corneal extract made from eyes of cow embryos, no observable improvement in growth resulted. In the case of epithelium, it was found that increasing the amount of aspartic acid (Parshley and Simms, 1946) in synthetic medium No. 199 from 60 mg. to 150 mg. per 1,000 ml., increased the amount of outgrowth and maintained it in good condition for a longer time.

c. *Natural media*

None of the semisynthetic media which were used gave as good growth of epithelium as natural media. The natural medium giving best growth consisted of 30-percent horse serum and five-percent chick-embryo extract in Earle's salt solution. Unfortunately this medium soon imparted a cloudiness to the plasma clot and the cells themselves became very granular. It was found, though, that once a good growth was established, a change could be made to the semisynthetic medium containing two-percent horse serum, and the clot and cells soon became clear. As far as could be determined, this semisynthetic medium with the high concentration of aspartic acid maintained epithelium as long as did the natural medium and in a far better condition for observation. For transplanting epithelium, the natural medium was again found to be better for encouraging growth.

Beef aqueous humor alone was found to be a poor medium. When horse serum and chick-embryo extract were added to it in varying amounts, there was some improvement, but this was not very great.

d. *Hydrogen-ion concentration*

The effect of pH on growth was also investigated. Synthetic medium No. 199 has a high pH of 7.6 to 7.8, whereas the complex medium has a lower pH of 7.2 to 7.4. To test for the effect of pH on growth, cultures were set up in a range of pH which varied from 7.0 to 7.8. The range was obtained by gassing the semisynthetic medium with the standard gas mixture of eight-percent carbon dioxide, 21-percent oxygen,

and 71-percent nitrogen. It was found that the corneal cells were very insensitive to alkaline conditions but were sensitive to acid, so that when the pH dropped below 7.0 as a result of metabolism, there was a visible effect on the cells. Therefore, as a standard procedure, the cultures were started with a high pH to allow for this drop. The fluid medium was changed twice a week.

Human cornea

Most of the findings with monkey cornea were readily confirmed with human cornea. However, a number of other variables had to be taken into account in the case of human tissue. The monkeys had all been healthy juvenile animals, and immediately after death the cuttings were taken in phosphate-buffered saline (Dulbecco and Vogt, 1954), and the cultures were set up shortly thereafter. With the human tissue, a number of uncontrollable variables were encountered, such as the cause of death, the age of the individual, the number of hours between death and removal of the tissue, and the number of hours the tissue was stored before setting up the culture. All these factors were recorded for each culture and later the results were analyzed with these variables in mind. A few examples of the results obtained from cultures set up from 63 individuals are given in Table 1.

1. *Epithelium*

Table 1 shows that there was a very great variation in amount of epithelial growth. As far as could be determined, the most important variable affecting the growth of epithelium was the age of the individual, contrary to the finding of Thygeson (1939). Tissue taken from young children or babies grew much better than that from adults. The time to the post mortem did not affect the growth very much. For example, fair epithelial growth was obtained from cuttings taken 20 hours after death. However, best growth was obtained with tissue taken eight hours or less after death. The length of storage of the tissue in phosphate-buffered saline was not critical within 24 hours, as Thygeson (1939) reported. As can be seen from the table, excellent growth was obtained after storage of cornea for 24 hours at 4°C. The cause of death was only an important factor when the final stages were prolonged, and then the epithelial growth was not very good. However, tissue taken from three individuals who died of cancer grew very well.

No human epithelium has yet been trans-

TABLE 1

Growth of human cornea in tissue culture

Age of Individual	Hours to Autopsy[1]	Hours Tissue Stored	Epithelium	Fibroblasts	
			Amount of Growth	Time Growth Started (days)	Time to First Subculture (days)
2 yr.	8½	24	****	22	17
1 day	13½	2	***	14	19
2 mo.	3	4	**	9	19
10 mo.	20	5½	**	11	
69 yr.	9½	4	*		
55 yr.	3	24	—		

[1] Corneal cuttings were taken during autopsy.
****—excellent growth, covering entire bottom of Carrel flask.
***—good growth, 8 to 10 mm. from edge of tissue.
**—fair growth, 3 to 4 mm. from edge of tissue.
*—poor growth, 1 to 2 mm. from edge of tissue.
— —no growth in 10 days.

planted, since the successful technique with monkey cornea has not yet been applied to the human. This method of transplantation will probably be successful, and it is anticipated that strains of epithelial cells will be maintained in tissue culture indefinitely.

2. *Fibroblasts*

Fibroblasts grew readily, but the time of appearance varied greatly. It can be seen from Table 1 that the better the epithelial growth, the longer it took the fibroblasts to appear. In one case the time was 22 days. The fibroblasts overgrew the bottom of the flask in an average of 18 days after they started to grow, and were transplanted at that time. A number of strains of fibroblasts are now being maintained in roller tubes and Carrel flasks.

Summary

1. The best epithelial growth from both human and monkey cornea was obtained using large thin corneal cuttings with an intact epithelial surface implanted up on a very shallow plasma clot and covered with a fluid medium composed of 30-percent horse serum and 5.0-percent chick-embryo extract in Earle's salt solution.

2. Human corneal epithelium grew quickly but there was a great variation in amount of outgrowth. In general, the best growth was obtained using tissue from babies or young children taken as soon after death as possible.

3. Pure cultures of corneal epithelium were obtained by removing the original explant of tissue before fibroblasts appeared.

4. Monkey corneal epithelium was subcultured using the enzyme trypsin to break up the original sheet when maximum growth was reached.

5. Good growth of fibroblasts was obtained from human and monkey cornea using either chopped or whole cuttings of cornea, implanted in a shallow plasma clot, and covered with a fluid medium composed of two-percent horse serum and 0.5-percent chick-embryo extract in synthetic medium No. 199.

6. Corneal fibroblasts grew slowly, taking from three to 22 days to appear, and from three to four weeks to overgrow the bottom of a Carrel D 3.5 flask.

7. Human and monkey fibroblasts were easily subcultured by scraping out the cells and clot and implanting small pieces in new flasks.

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TWENTY-THIRD MEETING

of the

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Proceedings

San Francisco, California

June 23-25, 1954

BUSINESS SESSION

CHAIRMAN MCDONALD: We will now hold the annual business meeting of the Association for Research in Ophthalmology.

We are sorry that there was some confusion in our plans, and that the dinner which was originally scheduled for tonight had to be held on Tuesday evening before some of you had arrived. The dinner was small in number but very select, and I think you should know, if you have not seen it in the papers, that Dr. David Cogan was given the Proctor Medal for this year.

There were two other presentations, that are not listed on the agenda, which I would like to tell you about. One was a silver platter given by the association to Dr. Brittain F. Payne for his many years as secretary and later as trustee.

The trustees themselves got together and presented this gavel to the association. We have never had a gavel of our own, and this was given to the association in recognition of the many years of service that Dr. James H. Allen has given as secretary-treasurer.

As you know, our attendance has increased and our membership has doubled during Dr. Allen's tenure in office.

We will now have the report of the secretary-treasurer, Dr. Allen.

DR. ALLEN: (Dr. Allen read the attached summary of the financial status of the organization. [See pages 249-252.]

At this time I would like to commend the work of the assistant secretary and the secretaries of the various sections. The sections have improved in their activities, and many of the their programs are now excellent and would serve as very fine programs for the national meeting.

The development in ophthalmic research around the country is becoming manifest by the number of papers being presented both at the sectional meetings and at the national meeting. It is very encouraging.

I would like to emphasize that membership in the Association for Research in Ophthalmology is not limited to physicians, but is open to anyone interested in research in ophthalmology or any aspect of vision and visual function.

CHAIRMAN MCDONALD: Are there any questions concerning this report? If not, the report will be accepted.

We will now hear the report of the auditing committee by Dr. Hughes.

DR. HUGHES: A complete audit was made by B. B. Woolley & Company in New Orleans, a certified public accountant organization, and Dr. Paul W. Miles and I looked over the report and found everything in order.

CHAIRMAN MCDONALD: Thank you, Dr. Hughes. Are there any questions concerning this report? If not, it will be accepted.

Before giving the report of the nominating committee, I would like to say that the trustees of this association are elected for a period of six years. In your senior year you act as chairman of the section. Like soldiers, you do not die—you just fade away. So, every year, we elect one trustee to the organization.

This year the nominating committee, composed of Dr. McGavic, Dr. Leopold, and Dr. Scheie, have nominated Dr. James H. Allen as trustee and Dr. Lorand V. Johnson as secretary-treasurer.

Do I hear any nomination from the floor? If not, the chair will entertain a motion that these men be elected to the office for which the nominating committee has recommended them. May I hear such a motion?

DR. COGAN: I so move.

DR. BRECHER: I second the motion.

(The motion was put to a vote and was carried unanimously.)

CHAIRMAN MCDONALD: The chair declares them elected.

Is there any further business to come before the association? If not, I declare the executive session closed.

AUDITORS' REPORT

Association for Research in Ophthalmology, Inc.

To the Members of the Board of Trustees
Association for Research in Ophthalmology, Inc.
New Orleans, Louisiana

Gentlemen:

Scope of Examination

We have examined the cash basis accounts of the secretary-treasurer of the Association for Research in Ophthalmology, Inc., as of December 31, 1953, and the recorded transactions for the year then ended. Our examination was made in accordance with generally accepted auditing standards applicable to cash basis accounting and accordingly included such tests of the accounting records and other such auditing procedures as we considered necessary in the circumstances, except that we did not confirm unpaid dues directly with members.

History

The Association was incorporated on July 20, 1936, under the laws of the state of New York. However, it had been an unincorporated group for some years earlier, operating under a constitution and related by-laws, which were embodied in the certificate of incorporation. The corporation has no shareholders and is exempt from federal, state, and local taxes. However, it is required to file a federal information return reporting the source and disposition of income annually.

Comments

At the meeting of the board of trustees in June, 1952, the board directed that the $250.00 annual interest income from the Proctor Medal Fund be deposited henceforth in the general fund and that the cost of the annual Proctor medal be paid out of the general fund.

According to information given us orally by the secretary-treasurer, it is the intention of the board of trustees, governing body of the association, to retain members whose dues are delinquent until the board itself orders that they be dropped for non-payment. The constitutional provision remains unchanged which provides that new members, upon being approved by the admissions committee and the board of trustees, must pay dues for the first year to attain membership.

At its meeting in May, 1953, the board of trustees directed that members whose dues were in arrears be notified and that if payment was not then forthcoming they be dropped from the rolls.

We did not verify by direct communication with members the dues which were delinquent. According to the records of the association, the entries therein showed 176 delinquencies as of December 31, 1953. If any members carried as delinquent had paid before that date and the payments had been lost in the mails before recordation, our examination might not have detected the omission.

All individual membership cards were examined and listed by us and their totals by classes of membership related to the recorded cash receipts for the period.

We found the records well kept. A number of improvements were inaugurated during the year as per our recommendations at the close of the preceding examination of accounts by us.

Certificate

In our opinion, subject to the limitation that we did not confirm dues in arrears directly with members, the accompanying statements present fairly the fund balances of the Association for Research in Ophthalmology, Incorporated, as of December 31, 1953, and the receipts and disbursements for the year then ended, based on the recorded cash transactions. The statements conform to generally accepted cash basis accounting principles applied on a basis consistent with that of the preceding year. We do not feel that the exception referred to above is sufficiently important to negate this opinion.

Very truly yours,
B. B. Woolley & Co.,
New Orleans, Louisiana,
June 21, 1954.

Cash and Securities in Funds
December 31, 1953

	General Fund	Proctor Medal Fund	Total
Cash:			
Cash in bank	$1,623.95	$ 15.37	$ 1,639.32
Securities:			
U. S. Treasury Bonds[1]	—	10,184.63	10,184.63
	$1,623.95	$10,200.00	$11,823.95

[1] At cost—2½%—due 1967-72. At December 31, 1953, coupons amounting to $250.00, matured in 1953, were affixed to the bonds, which were examined by auditors.

Statement of Cash Receipts and Disbursements
Year Ended December 31, 1953

		General Fund	Proctor Medal Fund
Cash Balance January 1, 1953		$1,973.52	$15.37
Add—Receipts:			
Prior years' dues:			
52 Active members @ $5.00 each	$260.00		
18 Educational members @ $2.00 each	36.00		
2 Sustaining members @ $25.00 each	50.00	346.00	
1953 Dues—(Page 5)		3,220.00	
1954 Dues—3 active members		15.00	
1955 Dues—1 active member		5.00	
1956 Dues—1 active member		5.00	
Banquet proceeds		450.00	
Bond interest (1952 interest deposited in bank in 1953)		250.00	
Total Receipts		4,296.00	-0-
Unidentified receipts		5.00	
		6,269.52	15.37
Deduct—Disbursements:			
Convention expenses:			
Dinners	$803.82		
Programs, mailing fees, notices	301.40		
Expenses of Secretary-Treasurer	250.00		
Other expenses	52.02	1,407.24	
Publication—American Journal of Ophthalmology		1,000.00	
Stationery, supplies and printing		478.69	
Auditing		155.00	
Postage		37.64	
Insurance—$5,000.00 position bond—Secretary-Treasurer		25.00	
Safety deposit box rental		9.60	
Telegrams		9.40	
Salaries—gross	650.00		
Less withholding and FICA taxes withheld	139.75	510.25	

	General Fund	Proctor Medal Fund
Withholding and FICA taxes paid	138.00	
Proctor Medals	[1]293.80	
Bank charges	2.37	
Contributions	[2]575.00	
Sundry	18.95	
Total Disbursements	4,660.94	–0–
Cash Balance December 31, 1953 (all deposited in bank)	$1,608.58	$15.37

[1] Proctor medals for 1952 and 1953 are included in this amount.
[2] Includes $400.00 to National Committee for Research in Eye Diseases and Disabilities. Board had authorized $500.00.

Changes in Membership and Reconciliation with Dues Paid

Year Ended December 31, 1953

Changes in Membership	Inactive	Life	Honorary	Educational	Active	Sustaining	Total
Membership—January 1, 1953	1	1	12	87	538	33	672
Add:							
Elected for 1953 membership at October, 1952, meeting of Board of Trustees				2	15		17
Elected for 1953 membership at June, 1953, meeting of Board of Trustees				14	15		29
Total additions	–0–	–0–	–0–	16	30	–0–	46
Deduct:							
Deceased in 1953			1		2	1	4
Resigned in 1953					1		1
Total deductions	–0–	–0–	1	–0–	3	1	5
Changes in Classes of Memberships:							
From active to sustaining					(7)	7	
From sustaining to active					9	(9)	
From educational to active				(21)	21		
From educational to sustaining				(2)		2	
From active to honorary			1		(1)		
Net changes	–0–	–0–	1	(23)	22	–0–	
Membership December 31, 1953	1	1	12	80	587	32	713
Reconciliation with Dues Paid:							
Dues Paid in 1952 for 1953					1		1
Dues Paid in 1953 for 1953:							
Inactive, life and honorary-waived	1	1	12				14
Educational @ $2.00 each ($ 60.00)				30			30
Active @ $5.00 each (2,310.00)					462		462
Sustaining @ $25.00 each (850.00)						34	34
	$3,220.00	1	1	12	30	463	34	541

	Inactive	Life	Honorary	Educational	Active	Sustaining	Total
Add:							
Unpaid memberships December 31, 1953 ...	-0-	-0-	-0-	50	126	-0-	176
	1	1	12	80	589	34	717
Less:							
One active member who resigned in 1953 but paid 1953 dues					1		1
One sustaining member who died in 1953 but paid 1953 dues						1	1
Unaccounted for difference					1	1	2
	1	1	12	80	587	32	713

Summary of Membership by Years

To December 31, 1953

Years Ended December 31	Total Members	Years Ended December 31	Total Members
1953	[1]713	1941	279
1952	[1]672	1940	270
1951	[2]556	1939	268
1950	[2]509	1938	272
1949	[2]474	1937	249
1948	422	1936	240
1947	306	1935	245
1946	324	1934	230
1945	[3]___	1933	219
1944	283	1932	203
1943	[3]___	1931	193
1942	281	1930	134

[1] Represents official membership, whether dues were paid or unpaid for current and prior years.
[2] Includes only members whose dues were paid in full to date.
[3] Not available, due to wartime dislocation.

OFFICERS

1954

Association for Research in Ophthalmology, Inc.

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P. Robb McDonald, Chairman ..... Philadelphia, Pennsylvania
William F. Hughes, Jr. ..... Chicago, Illinois
William B. Clark ..... New Orleans, Louisiana
T. E. Sanders ..... Saint Louis, Missouri
Michael J. Hogan ..... San Francisco, California
Hunter H. Romaine ..... New York, New York

Secretary-Treasurer

James H. Allen ..... New Orleans, Louisiana

Assistant Secretary

Lorand V. Johnson ..... Cleveland, Ohio

Section Secretaries

Eastern Section
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East Central Section
V. Everett Kinsey ..... Detroit, Michigan

Midwestern Section
Frank W. Newell ..... Chicago, Illinois

Southern Section
A. E. Meisenbach, Jr. ..... Dallas, Texas

Western Section
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V. Everett Kinsey
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Moacyr Alvaro

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Henry Imus
Kenneth N. Ogle

Committee on Local Arrangements
Michael J. Hogan

Committee on Study of Retrolental Fibroplasia
Leonard Christensen

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See key to references on next page.

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[1] Attended 1954 Meeting
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[3] Life Member
[5] Sustaining Member
[6] Educational Member

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